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Effect of Hypothalamic Stimulation on the Adrenaline Sectreion of the Adrenal Glands in Cats*

By

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The function of the hypothalamus on the central regulation of the sympathetic activity has been widely studied. Nevertheless, only a few experimentalists tried to estimate the adrenaline secretion rate during the hypothalamic stimulation.

Using the suprareno-jugular anastomosis, Houssay & Molinelli[1] stimulated electrically the infundibular portion of the hypothalamus and observed increases of the adrenaline secretion, the blood pressure and the rate of denervated heart in recipient dog.

In cats anesthetized with curare, Karplus & Kreidl[2] could not obtain any clearcut evidence concerning the share of the augmented adrenaline secretion of the adrenal gland in the blood pressure elevation after hypothalamic stimulation. Further, they compared in cats the adrenaline content of the adrenal gland of one side, removed before hypothalamic stimulation, with that of the other side, which was extirpated after the stimulation. However they could not find any significant difference between the estimates.

Van Bogaert[3] took it as highly probable that the hyperglycemia after stimulation of hypothalamus may be due to the acceleration of adrenaline secretion thereby produced, since it is abolished after decapsulation.

Using the cats anesthetized with dial, Magoun, Ranson & Hetherington[4] observed the liberation of sympathin and adrenaline after stimulation of the hypothalamus at the lateral hypothalamic or the perifornical area. In their experiments the contraction of the chronically denervated nictitating membrane of the same cats was used as an indicator of the sympathin liberation and the contraction of the acutely denervated one as an indi-

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cator of the adrenaline secretion from the adrenals.

In cats under light nembutal anesthesia, the hypothalamic stimulation experiments were performed by Bender & Weinstein.[5] The dilatation of the completely denervated iris and the delayed blood pressure fall were used as indicators of adrenaline secretion. They observed the responses after stimulation of the hypothalamus in the region near the third ventricle, 1 to 4 mm. lateral to the midline and 6–8 mm. below the zero horizontal plane. The stimulation of the hypothalamus in the more lateral area was less responsive.

In the experiments of Magoun, Ranson & Hetherington and of Bender & Weinstein, the adrenaline secretion was measured by the auto-assay-method, which is nowadays without specificity for estimating the adrenaline secretion from the adrenal glands.

In the present investigation we have undertaken to know the effect of hypothalamic stimulation on the adrenaline secretion of the adrenals by estimating directly the rate of secretion.

During describing this paper, we found the information of Brauner *et al.*,[6] who stimulated the hypothalamus near the tuber cinereum in cats anesthetized with chloralose. They estimated the adrenaline content of adrenal vein blood by testing it on the isolated hairs of the tail in cats and the isolated uterus of rats. The augmentation of adrenaline content in the blood taken during stimulation was detected: the maximum increase was from 0.08 μg. to 1.3 μg. per cc. of blood specimen. In regard to the rate of adrenaline secretion, however, no description was found in their paper.

Method

Cats, anesthetized with evipan-sodium, were used. For collecting the adrenal vein blood the following procedure was employed.

The right renal artery and vein were prepared through the lumbar route and were ligated. The lumbar vein of the same side was tied in the periphery of the adrenal gland. A silk thread was passed around the posterior cava at the site somewhat anterior to the entrance of the right adrenal vein. Then the left renal artery was tied. The left renal vein was tied at a distance of about 2 cm. from its opening into the posterior cava and clamped near the entrance. A small cannula for collecting the adrenal vein blood was inserted in the left renal vein just peripheral to the clamp. The left lumbar vein was also tied in the periphery of the adrenal gland. Before the start of experiment the abdominal aorta and the posterior cava were tied at the site somewhat below the left renal vessels. At the time of the collection of the adrenal vein blood specimen the silk thread passed around the posterior cava was pulled

gently and the clamp on the left renal vein was removed.

The adrenaline content in the adrenal vein blood was estimated by the rabbit intestine segment method and in some cases by the Bloor & Bullen's arseno-molybdic acid method.[7]

The blood pressure was recorded from the carotid artery using the Hg-manometer.

The fine bipolar electrode was inserted in the hypothalamus with the aid of the Horsley-Clarke stereotaxic instrument, and the current was supplied by the stimulator of thyratron-condenser circuit (Schmitt & Schmitt[8]). The frequency of the current was about 20 per second and the duration of stimulation was 60 seconds.

At the end of the experiments the brain was removed and preserved in formalin solution for examining the seat of the stimulation.

Results

The blood pressure

An abrupt rise of the blood pressure was observed just after the start of stimulation. Soon it reached its maximum and continued its height in most of the cases till the end of stimulation. With the cessation of stimulation the blood pressure began to descend and often reached below the initial height. The magnitude of blood pressure elevation was large when the ventral region of the hypothalamus was stimulated.

In Cat 4 (2.64 kg. ♀), after stimulating the ventro-medial region of the hypothalamus just lateral to the third ventricle at the level of the tuber cinereum, the blood pressure elevated from 98 mm. to 163 mm. Hg. By stimulating about 3 mm. lateral to the former in the same cat the blood pressure elevation of almost the same magnitude could be observed. In this case the blood pressure rose from 77 mm. to 159 mm. Hg.

In Cat 5 (3.14 kg. ♂), the seat of the stimulation was at the ventral area of hypothalamus and about 2 mm. lateral to the midline at the level just caudal to the caudal border of the optic chiasma. The blood pressure elevated soon after the start of stimulation from 76 mm. to 140 mm. Hg.

Almost the same area at the level of mamillary bodies was stimulated in Cat 7 (2.54 kg. ♀). By the stimulation the blood pressure rose from 92 mm. to 172 mm. Hg. The same portion was again stimulated in this cat and the elevation of blood pressure from 90 mm. to 160 mm. Hg was observed.

At the level of the caudal end of the hypothalamus the ventro-medial portion was stimulated in Cat 3 (3.15 kg. ♂). In this cat the blood pressure rose from 115 mm. to 177 mm. Hg. The blood pressure tracing

from this cat is reproduced in Fig. 1.

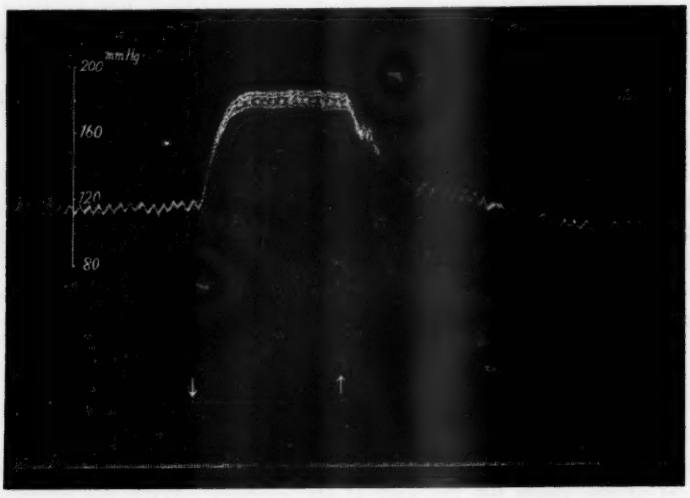

Fig. 1. Blood pressure tracing of Cat 3. Reduced to 1/2.
Stimulation of the ventro-medial hypothalamic area at the level of the caudal end of hypothalamus (↓ ↑). Time in second.

In Cat 6 (3.30 kg. ♀) the ventro-lateral area at the level of the tuber cinereum was stimulated at first. The blood pressure elevated from 64 mm. Hg and the height of 140 mm. Hg was reached. About 2 mm. more lateral and somewhat dorsal portion in the same frontal plane was then stimulated. In this case the magnitude of the blood pressure elevation was small. It ascended from 70 mm. to 102 mm. Hg.

The dorsal hypothalamic region about 1 mm. lateral to the midline in the same frontal plane as above was stimulated in Cat 1 (3.65 kg. ♂). The blood pressure elevated from 58 mm. to 101 mm. Hg. In the same plane the dorso-lateral area (about 4 mm. lateral to the midline) was stimulated in Cat 2 (3.54 kg. ♂). The blood pressure elevation of a small scale such as from 92 mm. to 113 mm. Hg was observed in this cat.

The adrenaline secretion of the adrenal glands

The initial rate of adrenaline secretion in the present experiments was 0.03–0.11 μg. per kg. of body weight per minute.

With the adrenal vein blood specimen collected during the hypothalamic stimulation, the augmentation of adrenaline secretion was observed. However, in Cats 1 and 2, in which the hypothalamus was stimulated at the dorso-medial and the dorso-lateral portion respectively, only the

augmentation of a small scale was observed. In Cat 1, the initial rate of secretion was 0.11 μg. per kg. per min. and the rate of secretion during the stimulation was 0.36 μg. Soon after the end of the stimulation it resumed the initial rate. In Cat 2, the adrenaline secretion rate before stimulation was 0.03 μg. per kg. per min. and during the stimulation it was 0.1 μg. Just after the stimulation it returned again to 0.03 μg.

The ventro-medial area in the caudal end of hypothalamus was stimulated in Cat 3. The initial rate of adrenaline secretion in this cat was 0.03 μg. per kg. per min. During stimulation it increased to 0.48 μg.; just after the stimulation it was 0.05 μg. In Cat 4, the ventro-medial area of the hypothalamus was stimulated. The adrenaline secretion increased from 0.04 μg. to 1.2 μg. per kg. per min. and just after the stimulation it was 0.02 μg. In the same cat the ventro-lateral area was then stimulated. Before stimulation the rate of adrenaline secretion was 0.03 μg. per kg. per min. and during stimulation it reached 1.2 μg. Therefore we could not find any differences between the both responses.

At the level just caudal to the caudal border of the optic chiasma the portion about 2 mm. lateral to the midline was stimulated in Cat 5. In this case the initial rate of adrenaline secretion was 0.03–0.04 μg., and during stimulation it increased to 0.82 μg. Soon after the stimulation it resumed the initial rate.

The ventro-lateral hypothalamic portion at the level of the tuber cinereum was stimulated in Cat 6. The initial rate of adrenaline secretion in this cat was 0.1 μg. per kg. per min. During stimulation it increased to 3.0 μg.; just after stimulation it was 0.29 μg. About 2 mm. more lateral and somewhat dorsal portion was also stimulated in this cat. The augmentation of adrenaline secretion could not be observed during and after this stimulation.

At the level of the mamillary bodies the ventral area about 2 mm. lateral to the midline was stimulated in Cat 7. Before stimulation the adrenaline secretion rate was 0.08 μg. During stimulation it increased to 5.1 μg.; just after stimulation it was 0.13 μg. The same portion was again stimulated in this cat: During the stimulation it increased to 4.4 μg. and just after stimulation it was 0.32 μg.

Discussion

In the present investigation we could confirm the acceleration of adrenaline secretion induced by the hypothalamic stimulation. The rate of adrenaline secretion during the stimulation in the ventral hypothalamic area was 0.48–5.1 μg. per kg. per min. In the paper of Bender & Weinstein it was reported that the rate of adrenaline secretion caused by the hypothalamic stimulation might be of the order of 0.6 μg. per kg.

per min., which is approximately the same as ours. They determined this value indirectly from the amount of adrenaline necessary to reporoduce the dilatation of pupils and the fall of the blood pressure of the same magnitude as was caused by the hypothalamic stimulation. Houssay & Molinelli measured in one experiment the amount of adrenaline liberated by the hypothalamic stimulation as 0.015 mg. The amount of adrenaline liberation per kg. of body weight of the donor dog was about 0.6 μg. In the paper of Brauner *et al.* the maximum value of adrenaline content in the adrenal vein blood during stimulation was reported as 1.3 μg. per cc. If the blood flow of the adrenal glands of both sides per kg. per min. is taken as 1 cc., the maximum value of adrenaline secretion will be 1.3 μg. per kg. per min.; this harmonizes with the value of Bender & Weinstein and that of ours.

Although the prolonged response of the denervated nictitating membrane or the denervated iris was reported by Magoun, Ranson & Hetherington and Bender & Weinstein, in most of our experiments the rate of adrenaline secretion resumed the initial value soon after the cessation of stimulation.

The responsive points in the hypothalamus were found in our experiments to locate in the ventral hypothalamic region. The augmentation of adrenaline secretion could be caused by the stimulation of ventro-medial as well as ventro-lateral hypothalamic area. In this respect, our results coincide well with that of Bender & Weinstein. Such remarkable augmentation of adrenaline secretion evoked by the hypothalamic stimulation just lateral to the third ventricle, as was observed in the present experiments as well as in those of Bender & Weinstein, was not noted in the paper of Magoun, Ranson & Hetherington.

Summary

In cats, anesthetized with evipan-sodium, the hypothalamus was stimulated electrically, inserting the electrode with the aid of Horsley-Clarke stereotaxic instrument.

The adrenal vein blood specimens were collected without opening the abdominal cavity and the adrenaline content in the blood specimens was estimated. The blood pressure was recorded simultaneously.

By the stimulation of the ventro-medial as well as the ventro-lateral hypothalamic portion, the blood pressure elevated abruptly. The adrenaline secretion rate before stimulation was 0.03–0.1 μg. per kg. per min. and during stimulation it increased remarkably to reach 0.5–5 μg. per kg. per min. Soon after stimulation the adrenaline secretion rate resumed in most of cases its initial value.

The authors wish to express their thanks to Prof. M. Wada for his helpful advices during this work.

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Peroxidase and Yakriton

Dr. Sh. Yanagawa as the First to Notice Effect of Yakriton to Intensity Peroxidase; Preliminary Report

By

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At an early time of isolation of yakriton,[1] the detoxicating hormone of the liver, Yanagawa[2] (now practician in Pediatrics in Tokyo) was studying the effect of the hormone in our Laboratory. Occasionally he tried to test our peroxidase reaction[3] on leucocytes of rabbits injected with yakriton, though I thought at that time that he did it rather out of mere curiosity.

Now the peroxidase reaction of rabbit leucocytes is not so intense as in the human case. Yanagawa used to say to me that *rabbit peroxidase reaction would be intensified by yakriton injection.* At that time I did not pay the slightest attention to these works of his, thinking each time that it must be an accidental result and that the very rabbit he had happened to experiment on must be of strong peroxidase reaction (at that time I had had an only poor experience with yakriton as well as our peroxidase reaction.) I did not even try to see his blood specimens.

A few years after he left our Clinic, our investigation of the Arakawa reaction[4] had revealed that yakriton injection could make the reaction (normally) positive even without thiamine administration to athiaminotic lactant, because it would mobilize[5] the (otherwise unutilized remaining) thiamine within the body. And it was soon found out that also leucocyte peroxidase reaction of rabbit could be intensified (=in fact, normalized) by yakriton alone (even without thiamine).

In other words, thiamine will remove the peroxidase reaction inhibiting substance from blood, and yakriton acts in the same direction due to its thiamine mobilizing effect.

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Effect of Alloxan on the Adrenaline Secretion of the Adrenal Gland in Non-Anesthetized Dogs*

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Few investigations have been done on the effect of alloxan upon the adrenaline secretion of the adrenal gland, although it has been suggested that the acceleration of adrenaline secretion may be cuased by alloxan.

Hard & Carr[1] suggested this possibility from the histological change in the adrenal medulla after the injection of alloxan and from the resemblance of the fluctuation of blood pressure produced by alloxan to that by adrenaline.

In the same year Goldner & Gomori[2] observed in rabbits that the initial alloxan hyperglycemia was diminished or wholly abolished by the removal of the adrenals or the destruction of the adrenal medulla and assumed that the initial hyperglycemia after alloxan will be due to the augmented adrenaline secretion from the adrenals.

This evidence was confirmed by Brown, Corkill, Fantl & Nelson,[3] who described in their paper as follows: "We consider that alloxan, in common with several other compounds, such as cysteine, glutathione etc., produces a non-specific hyperglycemia by stimulation of the adrenal medulla."

In the experiment of Houssay & Rapela,[4] using suprareno-jugular anastomosis, it was shown that the blood pressure elevation produced by alloxan is not due to the augmented adrenaline secretion. However, we are still unaware of the details.

The present study was designed to know quantitatively the effect of alloxan on the adrenaline secretion in non-anesthetized dogs.

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* Reported at the XXVIII Annual Meeting of the Japanese Physiological Society, Tokyo, April 4, 1951.

Method

Experiments were performed in dogs. The adrenal vein blood was collected according to the method of Satake and his co-workers[5] whithout anesthetizing, fastening or opening the abdominal cavity. D_{11}-L_3 dorsal spinal roots had been sectioned under morphine-ether anesthesia about 3 weeks or more prior to the experiment. The rabbit intestine segment method was used for the estimation of adrenaline content in the collected adrenal vein blood specimens. Adrenaline hydrochloride (Sankyo) was taken as the standard. The blood sugar content was measured by the method of Hagedorn & Jensen. Alloxan monohydrate (Eastman Kodak) was dissolved in a physiological saline solution and injected in the saphenous vein in a dose of 100 mg. per kg. of body weight.

Results

Experiments in the present investigation extended for 7 to 30 hours. The data are given in the table.

In Exp. 1 (female dog, 12.6 kg.), the blood sugar content before alloxan was 0.119–0.125%. After alloxan it decreased at first and was estimated as 0.090% 40 minutes after injection. Then it increased step by step and 0.278% was reached 7 hours after alloxan, when the experiment was terminated. The adrenaline secretion rate remained almost unaltered during the whole course of experiment. Before injection it was 0.02 μg. per kg. of body weight per minute from the adrenal gland of one side and after injection it was 0.02–0.04 μg.

In Exp. 2 (male dog, 16.4 kg.), the blood sugar content decreased at first from 0.119% to 0.088% after alloxan and then began to increase. Its maximum value (0.179%) was reached 3 hours after injection. Then it decreased again and the lowest value was estimated as 0.063% 8 hours after alloxan. Thereafter it increased and 10 hours after injection the initial value was resumed. 19, 24 and 30 hours after alloxan the blood sugar content was found to increase definitely and estimated as 0.152%, 0.161% and 0.172%, respectively. The rate of adrnaline secretion before alloxan was 0.03–0.04 μg. per kg. per min. Till 7 hours after the alloxan injection, it was found to remain unaltered or increase insignificantly. With the specimen taken 8 hours after alloxan, when the blood sugar content showed its lowest value (0.063%), the accelerated adrenaline secretion such as 0.14 μg. per kg. per min. was estimated. There-after the adrenaline secretion rate diminished gradually.

In Exp. 3 (male dog, 11.6 kg.), the blood sugar content before alloxan was 0.111–0.113%. It increased gradually after alloxan and one hour after, its maximum value (0.177%) was reached. Then it decreased slowly

TABLE I

Adrenaline Secretion Rate and Blood Sugar after Intravenous Injection of Alloxan

No. of Experiment	Time	Time after alloxan injection	Blood flow of the adrenal gland per kg. per min. c.c.	Adrenaline in 1 cc. adrenal vein blood μg.	Adrenaline secretion per kg. per min. μg.	Blood sugar %	Heart rate per min.	Respiration per min.	Body temperature °C.	Room temperature °C.
Exp. 1	4:40 p.m.		0.24	0.1	0.02	0.119	150	20	39.4	19
	4:55		0.19	0.1	0.02	0.125	135	18	39.4	19
Dog ♀	5:07	Intravenous injection of alloxan (100 mg. per kg. of body weight)								
12.6 kg.	5:27	20′	0.21	0.1	0.02	0.101	153	12	39.4	19
	5:47	40′	0.27	0.1	0.03	0.090				
	6:07	60′	0.29	0.1	0.03	0.125	129	12	39.4	19
	6:37	90′	0.25	0.1	0.03	0.166	120	12	39.6	19
	7:07	2 hrs.	0.27	0.1	0.03	0.177	105	8	39.4	19
	8:07	3 »	0.19	0.2	0.04	0.191	114	10	39.6	19
	9:07	4 »	0.18	0.1	0.02	0.217	105	10	39.5	19
	10:07	5 »	0.21	0.1	0.02	0.236	147	10	39.9	19
	11:07	6 »	0.16	0.15	0.02	0.255	111	10	39.3	19
	0:07	7 »	0.09	0.3	0.03	0.278	96	8	39.2	19
Exp. 2	1:20 p.m.		0.62	0.05	0.03	0.119	86	16	39.3	14
	1:30		0.55	0.075	0.04	0.119	94	18	39.3	14
Dog ♂	1:40	Intravenous injection of alloxan (100 mg. per kg. of body weight)								
16.4 kg.	2:20	20′	0.93	0.05	0.05	0.088	106	18	39.8	14
	2:20	40′	0.73	0.05	0.04	0.090	124	18	39.8	14
	2:40	60′	0.78	0.1	0.08	0.097	122	18	39.3	14
	3:10	90′	0.59	0.1	0.06	0.135	84	12	39.7	14
	3:40	2 hrs.	0.63	0.1	0.06	0.152	92	16	39.5	14
	4:40	3 »	0.61	0.1	0.06	0.179	114	18	39.4	14
	5:40	4 »	0.64	0.1	0.06	0.139	96	18	39.1	14
	6:40	5 »	0.51	0.1–	0.05–	0.104	82	12	38.9	14
	7:40	6 »	0.55	0.15	0.08	0.081	124	12	38.6	13.5
	8:40	7 »	0.62	0.1	0.06	0.070	98	16	38.6	13.5
	9:40	8 »	0.46	0.3	0.14	0.063	106	14	38.4	13
	10:40	9 »	0.51	0.2	0.10	0.113	100	16	38.7	12.5
	11:40	10 »	0.45	0.1	0.05	0.117	106	12	38.9	12.5
	8:40 a.m.	19 »	0.56	0.1	0.06	0.152	130	24	38.8	12.5
	1:40 p.m.	24 »	0.26	0.1	0.03	0.161	102	12	39.1	13.5
	7:40	30 »				0.172	120	20		14
Exp. 3	2:35 p.m.		0.96	0.025–	0.02–	0.113	156	12	39.3	10
	2:50		0.78	0.025	0.02	0.111	146	18	39.2	10
Dog ♂	3:00	Intravenous injection of alloxan (100 mg. per kg. of body weight)								
11.6 kg.	3:20	20′	0.99	0.025	0.02	0.131	132	19	39.7	10.5
	3:40	40′	0.80	0.025	0.02	0.172	150	22	39.7	10.5
	4:00	60′	0.83	0.025	0.02	0.177	166	17	39.6	10.5
	4:30	90′	0.78	0.025	0.02	0.166	166	17	39.8	10.5
	5:00	2 hrs.	0.60	0.025	0.02	0.164	174	21	39.8	10.5

6:00	3 hrs.	0.57	0.025	0.01	0.157	158	18	40.0	10.5
7:00	4 "	0.65	0.05	0.03	0.127	138	17	39.8	10
8:00	5 "	0.57	0.05	0.03	0.111	174	19	39.2	10
9:00	6 "	0.66	0.05	0.03	0.086	180	15	38.8	10
10:00	7 "	0.57	0.1	0.06	0.074	190	12	38.8	10
11:00	8 "	0.57	0.3	0.17	0.070	166	10	38.5	10
12:00	9 "	0.48	0.3	0.14	0.068	160	13	38.3	10
1:00 a.m.	10 "	0.53	0.25	0.13	0.066	122	13	38.4	9.5
11:00	20 "	0.57	0.1	0.06	0.070	134	12	38.2	9.5

and the initial value was resumed 5 hours after injection. It decreased further step by step and 10 hours after injection it was estimated as 0.066%. 20 hours after alloxan it was 0.070%. The adrenaline secretion rate before injection was 0.02 μg. per kg. per min. It remained unchanged during the hyperglycemic phase. It increased slightly 7 hours after injection. 8 hour after alloxan, when the blood sugar content was measured as 0.070%, the adrenaline secretion rate was estimated as 0.17 μg. per kg. per min. This value lasted till 10 hours after alloxan. With the last specimen taken on the next day (20 hours after alloxan), it was measured as 0.06 μg. The time relation between the fluctuations of blood sugar content and the rate of adrenaline secretion in this experiment is shown in the figure.

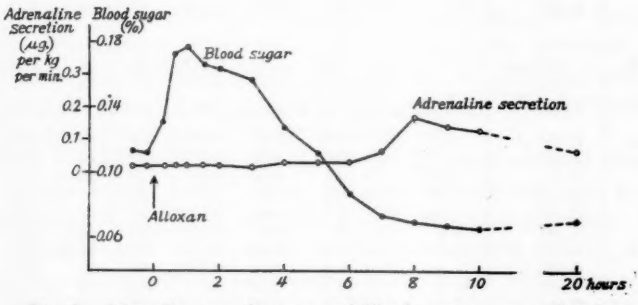

Fig. 1. Adrenaline secretion rate and blood sugar content after intravenous injection of alloxan in Exp. 3.

Discussion

The results of our experiments indicate that the adrenaline secretion does not materially alter after the alloxan application until the blood sugar content has decreased remarkably. Thus we are not justified to accept the views of Goldner & Gomori and Brown *et al.* that the initial alloxan hyperglycemia is due to the augmented adrenaline secretion from the adrenal gland.

Contrary to the evidences of the above investigators, Houssay, Orias & Sara[6] observed the hyperglycemia after alloxan in adrenalectomized

dogs anesthetized with chloralose. Their data were criticized in the paper of Lukens.[7] Indeed, at a glance of their data, it is noticeable that the hyperglycemia after alloxan is small in its magnitude and their experimental results are undecisive.

In the experiments of Kirschbaum, Wells & Molander,[8] the blood sugar content after alloxan was estimated in two demedullated rats. In one rat a remarkable hyperglycemia was observed, however in another the blood sugar increased only slightly. On the other hand, they could never observe the hyperglycemia after alloxan in adrenalectomized rats. The authors inferred that the adrenal cortex rather than the medulla is concerned in the initial hyperglycemic response to alloxan.

The role of the adrenal medulla in the developement of alloxan initial hyperglycemia will be discussed in detail elsewhere by one of us (T.).

The augmentation of adrenaline secretion during hypoglycemic phase was observed in our experiments. The maximum value was 0.14 μg. in Exp. 2 and 0.17 μg. in Exp. 3. These coincided roughly with the values of Dogs 2, 3, 4 and 6 in the paper of Yen, Aomura & Inaba,[9] in which insulin was injected in a dose of 0.25, 1.0, 23.0 and 7.0 units per kg., respectively.

Summary

Sampling the adrenal vein blood in dogs without anesthetizing, fastening or evoking pain, the adrenaline secretion rate before and after alloxan was estimated. Blood sugar content was measured simultaneously.

After alloxan the blood sugar content increased definitely. This initial hyperglycemia was followed by the hypoglycemia. The adrenaline secretion rate remained unaltered until an intense hypoglycemia took place. Therefore we can not accept the view of some authors that the initial alloxan hyperglycemia is due to the augmented adrenaline secretion from the adrenals.

At the hypoglycemic phase a definite acceleration of the adrenaline secretion was observed. Its maximum values were measured as 0.14-0.17 μg. per kg. per min. from the adrenal gland of one side.

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Histochemical Study on the Phosphatase Distribution in Rabbit Bone Marrow Cells

By

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Since the introduction of histochemical methods for the demonstration of phosphatase activity by Gomori[1)2)] and Takamatsu,[3)] many investigations have been made on the distribution of this enzyme in various tissues under normal and pathological conditions, including those on blood and bone marrow. This author has also attempted to study the distribution of acid and alkaline phosphatases in bone marrow cells, in the course of histochemical studies on hemopoietical tissues. In this experiment, some findings were noticed which had been not so fully described in the previous reports on similar cells. The most noteworthy finding was the negative reaction of mitotic chromosomes in both acid and alkaline ranges, which was completely opposed to the opinions of many authors, such as Danielli and Catcheside,[4)] Kurgelis,[5)] Brachet,[6)] Rabinovitch and Andreucci,[7)] and Sulkin and Gardner.[8)]

In last few years, on the other hand, specificity of the reaction pattern obtained by Gomori-Takamatsu methods has been called in question, especially, several investigators deciding the postitive reaction in nucleus as nothing but an artefact. But whether chromosomes of the dividing cells are poor in phosphatase activity or utterly lack in it is still in question, as not being reported on these phenomena by any investigator. In this paper, specificity and significance of these reaction patterns are also discussed.

Experimental

Material and Methods

Normal rabbit bone marrow was made to imprints on slides and treated as described below. Sodium-beta-glycerophosphate was used as the substrate of incubation mixtures for both alkaline and acid phosphatases.

Alkaline phosphatase: Air-dried imprints were dipped in dilute alcohol-ether-celloidin solution for about 10 seconds without any preceding fixation.

Thus coated slides were kept on a slant for several minutes unitl the celloidin became half dried, and immersed in absolute alcohol for removal of remaining ether, and next in 90 and 70% alcohol to harden the celloidin. Moderate drying of coated celloidin prior to immersing into alcohol was essential to prevent the celloidin membrane from getting off during the following treatments. After washing in tap water, slides were then incubated in Gomori's revised substrate mixture[9] (pH 9.0–9.4, tested with thymol blue paper) for 3–24 hours at 37°C, and visualized according to cobalt-sulfide method. Some slides were counter-stained with methyl green.

Control slides were inactivated by means of fixation for at least 10 minutes with 0.5% mercuric chloride solution or Zenker-acetic, prior to incubation. Moreover, according to Danielli,[10] these inactivated slides were incubated in another substrate mixture containing a small amount of 3% hydrogen peroxide solution (final volume per cent was 5–10) instead of magnesium salt, in order to examine unspecific adsorption of calcium phosphate which may occur during the long period of incubation.

Acid phosphatase: Imprints were fixed for not over half minute in chilled acetone or 10% neutralized formalin or 10% neutralized formalin-saline, and rinsed in water. Then, slides were incubated for 3–24 hours at 37°C in the substrate mixture described by Rabinovitch *et al.*[7][11] Ph of the acetate buffer used was 4.4–4.8 (tested with brom cresol green paper). Visualization was carried out in dilute ammonium sulfide solution.

Control slides were inactivated as similar as in alkaline phosphatase. In order to examine unspecific adsorption of lead phosphate, following test introduced by Gomori[12] in his critical study on the alkaline phosphatase was applied in this case of the acid one. An inactivated slide and non-inactivated one were made up to a pair, being superimposed together face to face each other. Both opposite faces were separated by a narrow space supported with insertion of cover glass between the ends of the two slides. Such a pair was incubated for 15-24 hours in the usual substrate mixture.

Observations

Alkaline phosphatase

In briefly incubated slides, positive reactions were evident only in the cytoplasm of pseudoeosinophilic myelo- or metamyelocytes and polymorphonuclear granulocytes, while their nuclei remained unstained (Fig. 1). These reactions were diffused in the cytoplasmic backgrounds among the specific granules which were recognized as unstained round particles. Promyelocytes showed very slight tint in their nuclei and cytoplasm in some specimens incubated for 6 hours.

With prolongation of incubation period, being kept for more than 15 hours, juvenile cells were brought to be visible accompanying simultaneous appearance of positive reaction in almost all nuclei of all cell-series. Such a nuclear reaction was diffuse and even in its pattern, and

scarcely seemed to be located within any specific structures, such as chromatin or nucleoli. After prolonged incubation near to 24 hours, cytoplasmic reaction of pseudoeosinophilic granulocytes had become apparently slighter than that of briefly incubated ones, while the blackening of their rod-shaped or segmented nuclei had been intensified.

Large round-nuclear cells without cytoplasmic granulations were also visible as faint gray shadow-like figures in such slides. As nuclei of small erythroblasts (probably, polychromatic and orthochromatic ones) had shown occasional staining after such prolonged incubation, these round cells were supposed to include not only myeloblasts but also early erythroblasts. It is very difficult, however, to identify cytologically to what cell-series each of them belongs.

Mitotic figures of these blast-cells in division exhibited a characteristic reaction pattern as represented in Figs. 2 and 3. Their chromosomes either showed nothing of the reaction or, if any, only a minimal one forming a striking contrast to considerably intensified impregnation on the cytoplasm. It must be taken in consideration here, that the greater part of the nuclei of resting cells in the same slides show more or less certain positive reactions. These general reactions found in most of the nuclei result from the prolonged incubation kept for more than 15 hours, indispensable to visualize the mitotic figures as most of them belong to these so-called juvenile cells which were beforehand observed as large round-nuclear cells.

Eosinophilic granules showed entirely negative reaction standing out against the dark background as well as pseudeoosinophilic granules. It was always easy thanks to these distinct negative figures of the granules, to distinguish pseudoeosinophilic and eosinophilic promyelocytes and myelocytes from other cell-series. Unspecific (azurophilic) granules of promyelocytes and basophilic granules could not be identified in any of the slides.

Mature erythrocytes did not show any reaction.

Megakaryocytes represented only faint and diffuse tint in their nuclei and border zone of their cytoplasm when the slides were incubated for long period. Reaction of the platelets was not observed.

Reaction patterns of monocytes, lymphocytes, and plasma cells yet remain not clear, as they can not be distinguished respectively from each other, nor from other round-nuclear cells. At least, however, reaction of these cells was not observed in briefly incubated slides. Those of lymphocytes and lymphoblasts were also searched using the imprints obtained from mesenteric lymph node as the materials. They exhibited only a slight and diffuse reaction in their nuclei after long incubation.

Inactivated slides treated simultaneously did not show any sign of

reaction. Another control slides incubated in the substrate solution which contained hydrogen peroxide showed far different results. Namely, though occurrence of reaction was almost not to be discernible except slightest signs within 3–6 hours of incubation, but after 15 hours, all nuclei of all cell-series had been pretty blackened remaining their cytoplasm unaffected. In the very case, pattern of the nuclear stain conformed almost exactly to that of in May-Giemsa stained ones, increasing in its intensity as maturation of the nuclei in both of white and red cell-series. For instance, blackening of the pycnotic nuclei of normoblasts was most prominent as they are usualy. Mitotic chromosomes were stained infallibly, though their contour was not invaliably distinct. Coincident results between these controls and experimental cases were restricted to the fact that both of them were negative in the reaction of eosinophilic and pseudoeosinophilic granules. Impregnation in the cytoplasm occurred after more prolonged incugation near to 24 hours, being accompanied with simultaneous blackening of mature red corpuscles.

Acid phosphatase

In the reaction pattern of acid phosphatase, there was no great difference between each cell-type and cell-series. Generally, almost all of the nuclei were impregnated most intensely in somewhat slighter cytoplasm, except certain cytoplasmic constituents, such as eosinophilic granules. In this experiment, it has become questionable whether the nuclear pattern of acid phosphatase takes after that of conventional hematological stain as claimed by Rabinovitch and Andreucci.[7] Large round nuclei containing distinctly unstained nucleoli in them (probably the nuclei of myeloblasts, proerythroblasts, etc.) took the pattern of fairely fine granular in reaction which was apparently coincided to that of chromatin. But the granular reaction was distributed more dispersedly than the network of basichromatin, and never identified was the so-called "nucleolus associated chromatin," which is usualy stained with basic dyes and Feulgen technic. Moreover, the nuclei of granulocytes and late erythroblasts showed thread-like reaction which seemed to be located in nuclear sap or oxychromatin, but at least not in basichromatin. Such a thread-like reaction of nuclei was most remarkable in the acetone-fixed specimens (Figs. 4 and 5). Ohter formalin-fixed ones, however, also showed very similar reaction pattern when they were incubated briefly (Figs. 6 and 7).

Mitotic nuclei represented negative reaction, as well as in alkaline phosphatase, but the outline of individual chromosomes was much better preserved (Figs. 5 and 6). Most of the cytoplasm of these dividing cells were stained more intensely than those of the resting cells. Such a characteristic staining pattern was never altered in quality with choice of the fixatives and length of the incubation period. In thick portion, however,

or in surrounding zone around solid clot of tissue in the same slides, asters of dividing nuclei showed occasional diffuse brown tint, and moreover, some of them were impregnated deep brown-black prominently standing out against light brown cytoplasm.

In the case of the acid phosphatase too, the evidence of specific granules only makes it possible to make a discrimination between the so-called juveniles of granulocytic series and those of the other series. Eosinophilic granules were readily distinguishable by their intense reaction even in the slides incubated briefly (Fig. 7).

Cytoplasmic reactions of pseudoeosinophilic series were somewhat more variable. Most of the slides showed negative figures of the granules in slightly stained cytoplasm, while occsaional granular reaction in cytoplasm was also recognized in some specimens. Such a granular reaction, however, was found mainly in the cytoplasm of promyelocytes and myelocytes, though that of polymorphonuclear ones in the same slides was stained more diffusely. These granules were not apparently coincided with the unspecific granules in their number and locality, when compared with control slides stained by means of May-Giemsa method, being obtained from the same animal. Basophilic granules were not identified.

Mature red corpuscles were not stained.

Coarse and intense reaction of the nuclei of megakaryocytes was observable in every case, but the pattern was somewhat different from the structures of the Feulgen stained nuclei. Their cytoplasm also showed intense granular tinting (Fig. 8), but the distribution pattern of these granules and that of azurophilic dusts (Schridde's granulation) were not exactly identical. Reaction of platelets was not found.

As regards reaction patterns of lymphocytes, monocytes, and plasma cells, only lymphocytic series was to be distinguished from the latter two, using lymph node imprints as the materials. Positive reaction of lymphocytes and lymphoblasts was recognizable only in their nuclei, but none in their cytoplasm (Fig. 9). Pattern of their nuclear reaction was granular and somewhat aggregated, especially in the nuclei of mature cells. Monocytes and plasma cells were considered to present some reaction at least in their nuclei, probably in so similar a pattern to that of lymphocytes, as make it very difficult to differentiate each of them. Nuclei of fat cells and reticulum cells were also impregnated.

No sign of specific reaction appeared in the control slides incubated in usual manner. To the inactivated ones of the slides superimposed in pairs, on the other hand, took place certain adsorption of lead phosphate. After 15 hours of incubation, almost all cells had been stained brown-yellow, and the nuclei deeper than the cytoplasm, chromosomes being also stained with indistinct outlines, though general pattern of the

impregnation was considerably diffuse and hazy. After 24 hours, vigorous impregnation had been observed, strictly locating within basichromatin of nuclei. Thus, nuclear pattern of these slides became to have strong resemblance to that of conventional hematological stain, showing intense impregnation of mitotic chromosomes standing out in relief against practically unstained cytoplasm (Fig. 12). Comparing Fig. 12 with Figs. 4-7, differences between impregnation of chromatin caused by secondary adsorption of lead salt and granular or thread-like reaction due to enzymatic activity are very easily recognizable.

The other slides of such pairs, the active ones, showed noteworthy results. After 15 hours, they had represented the reaction pattern same with that of usual active slides, showing distinct negative figures of mitotic chromosomes. After 24 hours, difference between the pattern of the active slide and inactive one superimposed together had become minimal. Nuclear pattern approached to that of Giemsa stained ones, exhibiting either prominent or slight diffuse tint of asters of mitotic chromosomes (Figs. 10 and 11). Intensely stained asters stood out prominently against paler or negligibly stained cytoplasm (Fig. 11), and on the other hand, cytoplasm surrounding diffusely tinted asters showed moderate reaction (Fig. 10). Such cytoplasmic reactions were only a difference discriminating the active slides from the inactive ones of the same pair.

Staining pattern of dividing cells in the thick portion surrounding solid clot of tissue in the usual active slides has strong resemblance to that in the active slides of superimposed pairs described here. It may also be due to secondary adsorption took place in such a portion.

Discussion

Histochemical demonstration of alkaline phosphatase in bone marrow cells was made previously by Wachstein,[13] Wislocki and Dempsey,[14] Rheingold and Wislocki,[15] Plum,[16] Takeuchi,[17] and as for both acid and alkaline ones, exhaustive studies were made by Rabinovitch *et al.*[7][11] Moreover, Horii *et al.*[18] had made phylogenetical comparative study on the both phosphatase activities in blood cells obtained from various species. Among these many observations, there exists no great difference, and the results presented here are also not inconsistent in their principles with the precedings, except some points, especially concerning with acid phosphatase as described below.

Specific reaction in the cytoplasm of erythroblasts and plasma cells described by Rabinovitch and Andreucci,[7] and granular reaction localized in the Golgi-zone in lymphocytes observed by Rabinovitch *et al.*[7][11] and by Kazikawa[19] as well, could not be found here. Nuclear pattern in acid phosphatase in this experiment resembled rather to the preceding

observations of Rabinovitch, than those of his later study.

Except these little differences, the most outstanding fact in the present observations is the negative reaction of mitotic chromosomes in both acid and alkaline ranges. This finding is perfectly contradictory against almost all of the reports published before which described positive phosphatase activity in nuclei, especially against observations of some authors insisting on the positive reaction located in chromosomes of various tissue cells. For instance, Danielli and Catcheside[4] observed alkaline phosphatase activity situates in the Feulgen positive bands of the chromosomes obtained from salivary gland of *Drosophila*, and Krugelis[5] suggested the existence of desoxyribonucleotidase activity in the nuclei and chromosomes. Newman, Feigin, Wolf and Kabat[20] confirmed recently the existence of activity of a certain group of alkaline phosphatase in the nuclei, using various substrates, inhibitors, and accelerators.

In last few years, the specificity of sites of the reaction obtained by Gomori-Takamatsu method have been criticized exhaustively. In these critical studies, great importance has been attached to the posibilities of the diffusion of enzyme itself or reaction product (calcium phosphate or lead phosphate) and the secondary adsorption of them by cellular constituents. Above all, nuclear staining in the alkaline phosphatase was decided as an artefact by Gomori[21] and Novikoff.[22] Some attempts to prevent such a technical error were also tried by Bélanger[23] and Goetsch, Reynolds and Bunting,[24] both reports describing the negative reaction of nuclei.

Specificities of the results about acid phosphatase distribution also could not stay free from criticism. Noback and Paff[25] studied on the intracellular distribution of acid phosphatase activity in fibroblast, and found diffusion and shifting of positive reaction from cytoplasm into nuclei through nuclear membrane with prolongation of incubation period. Newman, Kabat and Wolf[26] demonstrated natural affinity of tissues to lead salt which was especially vigorous between pH 5.0–7.0. Attempts to check these artefacts were also made by Goetsch *et al.*[24] and Takeuchi and Tanoue,[27] and nuclei remained unstained in both of their experiments. This author[28] also observed previously the occurrence of lead salt precipitation on the smears and imprints of bone marrow when they were fixed with basic lead acetate, but it was seen to be strictly localized within basichromatin of nuclei. This is fairly agreed with the observation in this paper on unspecific impregnation occurred in the inactivated imprints with superimposing test according to Gomori.

The observations about absence or slightness of nuclear phosphatase activity, obtained histochemically as described, have many agreeing points with what has been introduced bio- and cytochemically with differential

fractionation of cellular constituents. Berthet *et al.*[29)30)] studied recently on the intracellular distribution of acid phosphatase in rat liver with such technic, and found out that the enzyme was naturally bound to mitochondria in non-active state, and when it was released out from the bond with mitochondria under various conditions, it acquired its whole enzymatic activity, and might be adsorbed secondarily in fully active state by various structural constituents, such as mitochondria, nuclei, and others. Such a migration of acid phosphatase itself may possibly occur in the conventional Gomori technic, because, according to Berthet's experiments, the enzyme-mitochondria binding is protected under O°C.

Concerning such doubtfull existence of phosphatase activity in nuclei, negative reaction of chromosomes is not to be regarded as so incomprehensible a phenomenon. It is noticed in this report, however, especially in the case of the alkaline phosphatase, that the negative reaction of mitotic figures is observed in such slides, in which almost other nuclei are stained considerably, as the positive reaction of these actively proliferating juvenile cells has been produced only after prolonged incubation over 15 hours, just as Takeuchi[17)] also observed. Perhaps in these slides, diffusion and secondary adsorption of calcium phosphate have taken place already, because reaction of the cytoplasm of polymorphonuclear granulocytes seems weaker in its intensity than that of briefly incubated slides, while the reaction of nuclei is brought to be visible. Such an inversion of the site of reaction during incubation is very similar to Moe's[31)] observations on the intestinal mucosa, which he has attributed to the diffusion of enzymatic product but not of enzyme itself.

There arises, therefore, an interrogation whether the diffusion of calcium phosphate from chromosomes causes such negative figures. This doubt may be resolved by taking into consideration the results of Danielli's test, which is supposed to reveal natural affinity of the cells to calcium phosphate. In this test, no difference of adsorbing affinity was found between resting nuclei and dividing ones. Moreover, appearance of adsorption in the cytoplasm occurred more later than in nuclei.

Educing from these results of Danielli's test, it is completely difficult to consider that adsorption of calcium phosphate takes place only in the cytoplasm of dividing cells, remaining their chromosomes unaffected, or that chromosomes represent less affinity to calcium phosphate than cytoplasm or resting nuclei. Accordingly, negative reaction of chromosomes indicates conceivably loss or decrease of enzymatic activity in nuclei during mitosis. Then it becomes not so unreasonable to consider that a certain portion of nuclear reaction is attributed to enzymatic activity, even though the greater part of it caused after long period of incubation may be due to unspecific adsorption of calcium phosphate.

In the case of the acid phosphatase, on the other hand, negative reaction of mitotic chromosomes is not a specific phenomenon restricted to dividing cells. Positive reaction in resting nuclei is dispersedly granular or thread-like in its pattern and is considerably located in nuclear sap or oxychromatin, but not in basichromatin. Negative reaction of chromosomes, therefore, may be regarded as an expression of natural absence of the acid phosphatase activity in chromatin.

Various staining patterns of dividing cells in active slides of the superimposed pairs are highly significant in their import. After 15 hours of incubation, they showed specific enzymatic activity alone, while another slides of the same pairs, the inactive ones, represented only slight and hazy impregnations. Reaction observed in the similar slides incubated for 24 hours was a total result of enzymatic activity and secondary adsorption. In these slides, nuclei and cytoplasm of the dividing cells showed various staining patterns, which were gradated into three orders as followings: slightly stained asters of chromosomes in moderately stained cytoplasm; moderately stained asters in moderately stained cytoplasm; and intensely stained asters in slightly or negligibly stained cytoplasm. And similar cells in the inactivated slides of the same pairs showed asters invariably stained intensely in unstained cytoplasm. Comparing with the natural affinity to lead salt in basichromatin as described previously by this author, such a gradation of impregnation described here is undoubtedly nothing but the representation of diffusing and shifting process of enzymatically produced hydrolysates. It is conclusive, therefore, that intense impregnation in acid range restricted to chromosome and chromatin described by Rabinovitch and Andreucci[7] is an artefact, and moreover, that the greater part of the positive reaction obtained with incubation within 15 hours may be regarded as the representation of the real site of acid phosphatase activity.

Another noteworthy finding is the considerably intensified reaction in the cytoplasm of the cells during mitosis, in both acid and alkaline ranges. This increased reaction in alkaline range may be attributed to increased enzymatic activity, educing from the results of Danielli's test, while the significance of that in acid range remain obscure, whether it is due to real increase of the enzymatic activity.

In the preceding paper,[32] this author noticed that, on the contrary to division of general somatic cells, intensity of cytoplasmic basophilia of the blast-cells of bone marrow had never decreased during mitosis. This fact is very interesting when compared with the increased alkaline phosphomonoesterase activity in the cytoplasm of the similar cells, which may suggest accerelated hydrolysis of ribonucleic acid. Accordingly, in the case of division of these blast-cells, it seems that vigorous synthesis of

ribonucleic acid may be simultaneously proceeding, while total ribonucleic acid content in the cytoplasm is not apparently altered. Significance of the decrease of alkaline phosphatase activity in the chromosomes is not clear.

Summary

Intracellular distributions of acid and alkaline phosphatase activities in bone marrow imprints obtained from normal rabbit were studied histochemically according to Gomori-Takamatsu methods.

The most outstanding result obtained in this experiment was that the negative reaction of mitotic chromosomes in both acid and alkaline phosphatases, which was accompanied with simultaneous intensification of reaction in the cytoplasm. Although such reaction patterns in both phosphatases are very similar, significances are different from each other.

Negative or minimal reaction of the chromosomes in alkaline phosphatase represents loss or decrease of enzymatic activity, and increased reaction in the cytoplasm of the same cell shows increased enzymatic activity. On the other hand, negative figures of mitotic chromosomes in acid reaction do not seem to be the specific phenomena restricted to cell division and should be interpreted as the expression of the natural absence of acid phosphatase activity in chromatin of nuclei.

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Note at proof-reading: After this manuscript had been submitted for publication, the writer received several papers dealing with nuclear phosphatase kindly sent from Drs. Richterich, Novikoff and Goetsch. Richterich[33] discussed on nuclear phosphatase with reference to many literatures, Novikoff[34)35)] described negative alkaline phosphatase reaction of nuclei of intestinal mucosa and mitotic chromosomes of regenerating liver, and according to Goetsch and Reynolds,[36] acid phosphatase is not detectable in cell nuclei.

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Explanation of Figures

Fig. 1. Alkaline phosphatase reaction after 6 hours of incubation. Positive reaction is restricted within the cytoplasm of polymorphonuclear granulocytes and myelocytes.

Figs. 2 and 3. Negative reaction of mitotic figures in alkaline phosphatase after 24 hours of incubation. Small spot in Fig. 2 shows a late erythroblast.

Fig. 4. Acid phosphatase reaction in acetone-fixed slide incubated for 24 hours, showing dispersedly granular and thread-like reaction in nuclei. Negative reaction of nucleoli is also visible.

Fig. 5. Similarly treated slide as the above, showing distinct negative figures of mitotic chromosomes.

Figs. 6 and 7. Acid phosphatase reaction in formalin-saline-fixed slide after 6 hours of incubation. Fig. 7 represents a dividing eosinophilic myelocyte containing intensely stained granules in its cytoplasm.

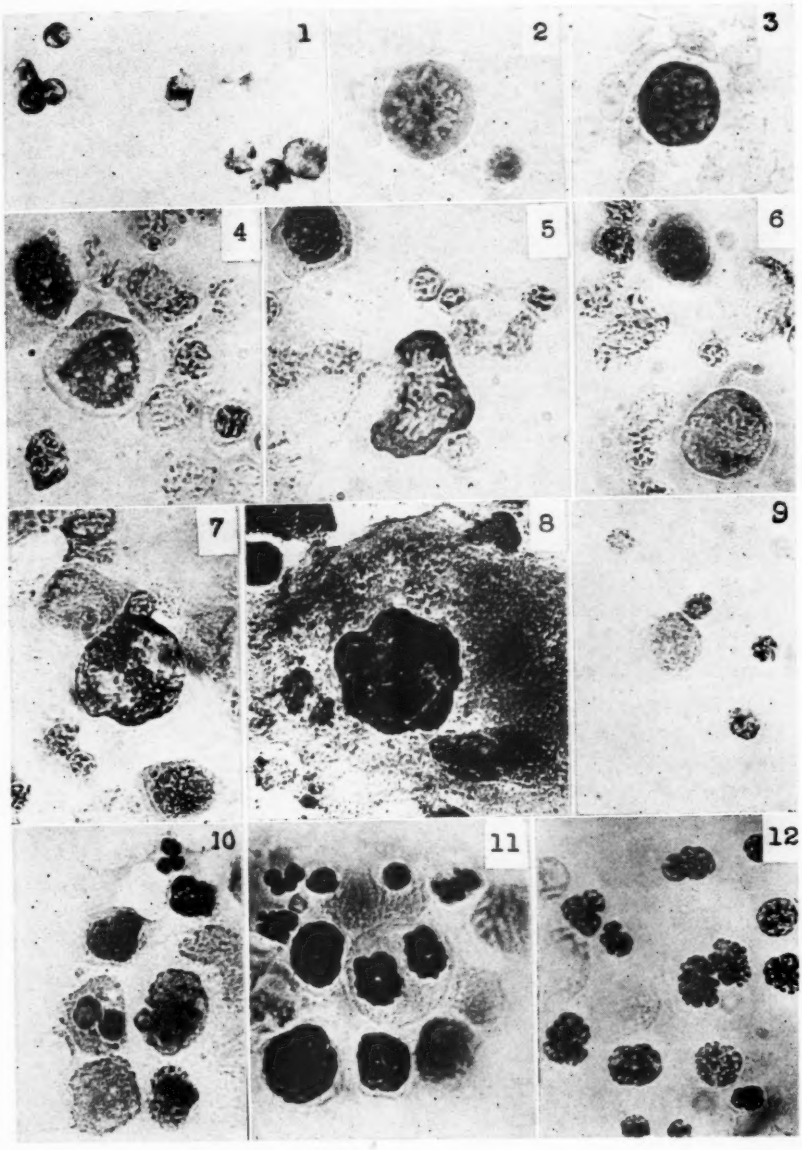
Fig. 8. Acid phosphatase reaction in nuclei and cytoplasm of megakaryocyte in formalin-fixed slide incubated for 24 hours.

Fig. 9. Acid phosphatase reaction in lymph node imprint fixed with formalin-saline, and incubated for 16 hours.

Fig. 10. Reaction in an active slide of a superimposed pair incubated for 24 hours in acid substrate mixture. Moderate and diffuse tint of mitotic asters is visible.

Fig. 11. Different portion from the same slide, showing intense impregnation of chromosomes and chromatic materials.

Fig. 12. Reaction in an inactivated slide of the same pair superimposed together with the slide represented in Figs. 10 and 11. Intense and distinct impregnation is restricted to chromatic materials.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Studies on Cholinesterase

III. On the Cholinesterase of Cerebrospinal Fluid in Various Diseases

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In the first paper, we reported the distribution of cholinesterase (ChE) in the human brain[1] and, in the second paper, the change of ChE in the human serum in various diseases[2]. In this paper, we will report on ChE variations in the cerebrospinal fluid. In comparison with ChE in the serum, the activity of ChE in the cerebrospinal fluid is so weak that its determination has never been performed until quite recent. Reiss and Hemphill reported in 1948 on ChE activity in the cerebrospinal fluid[3]. In order to make manometric determination possible, these authors used the extracted fluid without dilution, thus preventing decrease of ChE activity. We duplicated this method and confirmed its applicability to clinical research. Although Tower and McEachern[4], and Wollman[5] reported on studies on ChE in the cerebrospinal fluid, we do not seem to have any comprehensive reports on the variation of this substance in general neurological patients, and we are reporting in this paper on the values of ChE activity in the cerebrospinal fluid of 140 cases of various neurological patients.

Method

Most of the specimens of cerebrospinal fluid were obtained from in-patients, with the exception of a few cases where these were taken from out-patients of Prof. Okinaka's Clinic. All the material was taken from the patients before treatment, and that of patients whose diagnose were not certain was excluded. ChE activity was measured within one to three days, during which time the fluid was stored in a refrigerator, which was found to prevent any decrease of ChE activity.

The ChE values were determined on the method of Reiss and Hemphill, using Warburg's apparatus: 1.8 cc. of cerebrospinal fluid and

0.2 cc. of 2 per cent sodium bicarbonate solution was poured into the main body of the flask, while 0.2 cc. of 5 per cent acetylcholine solution was inserted in the side tube. The gas room was filled with nitrogen gas containing 5 per cent carbondioxide. After equilibrium was attained in the thermostat at 37.5°C, the acetylcholine solution in the side tube was run into the main body. Readings were taken 10, 20, 30 and 40 minutes after mixture. Production of CO_2 gas during this period proceeded at a constant rate. Autolysis of the acetylcholine was negligible and was therefore omitted from the calculations. ChE activity was expressed in cubic mm. of CO_2 produced per 1 cc. cerebrospinal fluid per 30 minutes.

Results

Values obtained are shown in the accompanying table and figure. Large deviations are observed, ranging from the minimum value of 4.8 in poliomyelitis to the maximum value of 182.0 in tumor of spinal cord. There proved to exist a definite relationship between the ChE activity of the cerebrospinal fluid and a number of diseases.

1. Normal persons

In 10 cases of healthy male and female subjects, from 2 to 61 years of age, the value of ChE in the cerebrospinal fluid was 16.9 on the average, with a range of 14.5 to 19.0. Difference due to sex or age was not observed.

2. Tuberculous meningitis

The average value of 16 cases was 39.8, showing marked increase of activity. Even in patients whose uranin test for meningeal permeability had been negative or slightly positive, an increased value of ChE in the cerebrospinal fluid was detected when measured before the occurrence of the change to the positive uranin test results.[7] It appeared that the level of ChE activity of the cerebrospinal fluid corresponded to the gravity of the prognosis of the patients.

3. Poliomyelitis

In all the cases except one, cerebrospinal fluid was taken at the initial stadium of the paralysis, Among 17 patients, 3 cases showed normal values while the remaining 14 cases showed decreased activity. The average value was 11.1.

4. Tumor of brain and spinal cord

12 cases of brain tumor averaged the heightened value of 35.2. The highest value of 151.0 was observed in a case of Hippel-Lindau's disease. All 6 cases of glioma showed increased values. In 4 cases normal or decreased values were obtained, namely, ependymoma, cholesteatoma, miningeoma and acidophil adenoma of the anterior pituitary. All 3 cases of tumor of spinal cord showed increased values, and averaged 107.2.

TABLE I

ChE Activity in Cerebrospinal Fluid of 140 Patients of Nervous Diseases

	Diagnosis	No. of cases	Average value	Minimum and maximum value
1	Normal persons	10	16.9± 1.41	14.5— 19.0
2	Tuberculous meningitis	16	39.8±12.4	19.3— 81.0
3	Poliomyelitis	18	11.1± 4.17	4.8— 18.8
4	Tumor of brain	12	35.2±36.3	8.0—151.1
5	Brain abscess	3	30.8± 6.76	22.6— 39.2
6	Cranial metastasis of gastric & esophag. cancer	3	15.4± 2.93	13.3— 18.8
7	Tumor of spinal cord	3	107.2±54.9	51.7—182.0
8	Adhesive arachnoiditis	3	11.8± 3.96	7.4— 17.0
9	Progressive paralysis	10	12.1± 1.6	9.0— 13.8
10	Genuine epilepsy	6	12.0± 2.17	9.1— 16.0
11	Hemiplegia after cerebral hemorrhage	6	19.0± 4.20	14.7— 26.1
12	Paralysis agitans	3	15.2± 6.74	8.3— 24.3
13	Chorea minor	3	12.7± 1.22	11.6— 14.4
14	Spastic spinal paralysis	2	19.9± 2.75	17.1— 22.6
15	Myasthenia gravis	2	11.9± 0.21	11.2— 12.5
16	Myelitis	3	18.6± 3.39	15.0— 17.7
17	Neural progressive muscle atrophy	2	15.3± 2.76	15.4— 19.2
18	Progressive muscular dystrophy	3	12.2± 5.16	12.0— 21.0
19	Polyneuritis	2	18.3± 1.55	16.7— 19.8
20	Syringomyelia	2	17·5± 0.50	17.0— 18.0
21	Herpes zoster	4	14.7± 2.51	14.1— 18.1
22	Diabetes mellitus	3	15.2± 3.91	11.9— 20.7
23	Commotio cerebri	1	25.6	
24	Periodic paralysis	1	21.3	
25	Liver cirrhosis	1	20.8	
26	Bronchial asthma	1	20.0	
27	Ischias	1	20.0	
28	Subarachnoidal hemorrhage	1	19.6	
29	CH_3Cl intoxication	1	19.0	
30	Erythromelalgia	1	18.3	
31	Myotonia congenita	1	17.8	
32	Schizophrenia	1	17.0	
33	Spontan nystagmus	1	17.0	
34	Atrophia nervi optici simplex	1	16.9	
35	Senile dementia	1	15.8	
36	Facial nerve paralysis	1	14.3	
37	Brain edema	1	13.9	
38	Idioty	1	13.4	
39	Neuritis axialis	1	12.5	
40	Pulsless disease (Buerger's disease)	1	12.0	
41	Subdural hemorrhage	1	10.9	
42	Hirschsprung's disease	1	9.5	
43	Acute yellow liver atrophy	1	5.4	

In comparison with the increased ChE value in patients of brain and spinal cord tumor, normal values were found in the following patients having symptom of brain tumor: 3 cases of adhesive arachnoidities; 3

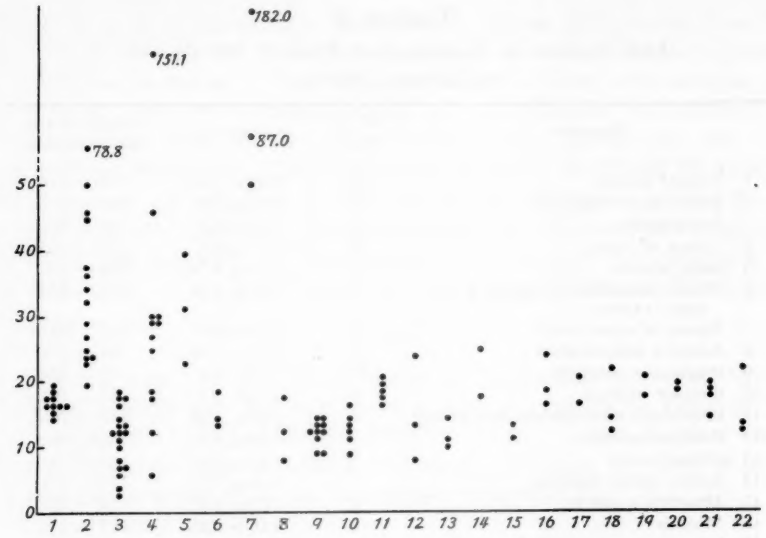

Fig. 1. ChE activity in cerebrospinal fluid of patients of nervous diseases. 1: Normal persons, 2: Tuberc. meningitis, 3.: Poliomyelitis, 4: Tumor of brain, 5: Brain abscess, 6: Cranial metastasis of gastric & esoph. cancer, 7: Tumor of spinal cord, 8: Adhesive arachnoiditis, 9: Progressive paralysis, 10: Genuine epilepsy, 11: Hemiplegia after cerebral hemorrhage, 12: Paralysis agitans, 13: Chorea minor, 14: Spastic spinal paralysis, 15: Myasthenia gravis, 16: Myelitis, 17: Neural progressive muscle atrophy, 18: Progressive mascular dystrophy, 19: Polyneuritis, 20: Syringomyelia, 21: Herpes zoster, 22: Diabetes mellitus.

cases of cranial metastasis of esophageal and gatric cancer. 3 cases of brain abscess showed increased values.

5. Progressive paralysis

Decreased values were found in all 10 cases, in which the cerebrospinal fluid showed strong positive reaction to globuline tests. The average value was 12.1.

6. Epilepsy

Decreased values were found in 5 out of the 6 cases of epilepsy. The average value was 12.0. Three samples of cerebrospinal fluid, taken 30 minutes after spontaneous fit, provoked fit with cardiazol and pumping respectively, showed no deviation from the normal value of ChE activity.

7. Hemiplegia after cerebral hemorrhage, paralysis agitans, chorea minor, spastic spinal paralysis, myasthenia gravis, periodic paralysis and myotonia congenita all showed values in or near the normal range.

Discussion

Although ChE activity of the cerebrospinal fluid is very weak, it was possible to measure the value quite accurately and detect deviations to it in various diseases. According to Tower and McEachern, ChE of the cerebrospinal fluid is chiefly of the " specific " or " true " type, as distinct from the " unspecific " or " pseudo " type to which serum ChE belongs. As already stated in this report and in our II report on serum ChE, the change of ChE values in the cerebrospinal fluid is completely independent of that of serum ChE. However, the increase observed in the ChE values of the cerebrospinal fluid in tuberculous meningitis, and tumor of brain and spinal cord, in contrast to the decrease of the same activity in the cases of progressive paralysis seems to indicate some relationship between ChE value and protein content, especially albumin content of the cerebrospianl fluid.

Measurement of ChE activity of the cerebrospinal fluid might be useful in differentiating tumors related directly to brain parenchyma accompanied by very high activity, from diseases showing similar clinical symptoms such as adhesive arachnoiditis or cranial metastasis of cancer, which usually show no appreciable increase of ChE activity.

Although the ChE activity of the cerebrospinal fluid may be clearly demonstrated, the activity is much weaker than that of parenchyma of the central nervous system. Davis reported the demonstration of acetylcholine in the cerebrospinal fluid by an acetone-sensitized frog rectus muscle test,[6] and Tower and McEachern reported to have found acetylcholine in the cerebrospinal fluid of patients with grand mal epilepsy[4]. According to our experinece, however, it would seem difficult to demonstrate acetylcholine in normal cerebrospinal fluid by using the eserinized frog rectus muscle. It seems to be inconceivable that ChE in the cerebrospinal fluid should play a part in acetylcholine metabolism in normal persons. Its physiological role appears to be insignificant in comparison with ChE in the nervous tissue. Change of ChE in the cerebrospinal fluid in patients of various diseases might be understood as the result rather than the cause of the symptoms of the disease.

Summary

1. ChE activity in the cerebrospinal fluid of 140 patients of various nervous diseases was measured manometrically, using Warburg's apparatus.

2. The value of ChE was expressed with CO_2 cubic mm. peroduced per 1 cc. cerebrospinal fluid per 30 minutes.

3. ChE in the cerebrospinal fluid is much weaker than in the serum or the brain tissue, and its value varies independently of change in the

serum ChE value.

4. Increased ChE activity of the cerebrospinal fluid was found in tuberculous meningitis and tumor of brain and spinal cord, while in poliomyelitis, progressive paralysis and epilepsy the value was found to be lowered.

5. No abnormal value of ChE in cerebrospinal fluid was confirmed in the following diseases: flaccid and spastic hemiplegia following cerebral hemorrhage, paralysis agitans, periodic paralysis, chorea minor, spastic spinal paralysis, myasthenia gravis, myotonia congenita, progressive muscular dystrophy, etc.

6. The physiological role of ChE in cerebrospinal fluid was discussed.

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Studies on Cholinesterase

IV. On the Relationship between Cholinesterase of Basal Ganglia and Liver Diseases

By

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In our previous study on the distribution of cholinesterase in the human brain,[1] we reported that basal ganglia have a larger content of ChE than the cerebral cortex and further that the putamen and caudate nucleus have an extraordinarily high concentration of ChE. In a later study on ChE of the human serum,[2] we confirmed the finding reported by many authors that serum ChE is decreased in patients of liver diseases. On the other hand, it is a well known fact that in Wilson's disease, liver cirrhosis and degeneration of lenticular nuclei are observed to occur simultaneously. This clinical experience suggests that there is some connection between basal ganglia and the liver. Our confirmation of the existence of a definite relationship between the serum ChE and the liver led us next to the consideration of the relationship between ChE of basal ganglia and liver diseases.

Method

14 patients of liver diseases and 14 cases of control diseases were examined. Gastric cancer was included in the group of liver diseases, since serum ChE in patients of that disease was found to decrease as markedly as with any other example of the group. Diagnosis was confirmed by autopsy. A sample of brain was taken out as early as possible, cut at the part of the corpus mamillare in the frontal direction, and putamen, caudate nucleus and pallidum were enucleated. These were cleaned with filter paper, weighed on a torsion balance and homogenized in Ringer's solution by using a Potter-Elvehjem homogenizer. The con-

TABLE I

Cholinesterase of Basal Ganglia in Patients of Liver Diseases

	Diagnosis	Sex	Age	ChE activity of basal ganglia*		
				Putamen	Caudate nucleus	Pallidum
1	Gastric cancer	Male	37	143.8	174.9	33.7
2	"	"	47	285.0	255.0	21.8
3	Gastric cancer with liver metastasis	"	59	275.0	216.9	79.6
4	"	"	48	198.9	171.2	—
5	Gastric cancer with biliary cirrhosis of liver	"	57	184.8	258.9	36.6
6	Liver cancer	"	56	132.5	199.2	—
7	Cancer of bile duct with liver cirrhosis	Female	40	311.7	192.0	40.6
8	Cancer of pancreas with liver cirrhosis	Male	66	228.0	213.0	44.6
9	Liver cirrhosis	"	75	—	213.8	125.0
10	Liver cirrhosis with liver cancer	"	50	245.0	202.9	—
11	Acute yellow liver atrophy	"	36	241.8	181.1	36.0
12	"	"	50	246.0	542.0	20.8
13	"	"	25	355.0	323.0	69.3
14	Hemachromatosis	"	52	129.0	86.0	23.4

TABLE II

Cholinesterase of Basal Ganglia in Patients of Control Diseases

	Diagnosis	Sex	Age	ChE activity of basal ganglia*		
				Putamen	Caudate nucleus	Pallidum
1	Tuberculous meningitis	Female	19	208.5	71.7	10.4
2	Miliary tuberculosis	"	44	193.6	111.0	20.0
3	Pulmonary tuberculosis with meningitis tbc.	"	36	203.0	132.5	31.8
4	Miliary tuberculosis with meningitis tbc.	Male	21	251.0	396.5	—
5	Cancer of the lung	"	75	186.3	95.4	14.7
6	Cancer of the lung with metastasis of brain	"	59	251.0	—	31.8
7	Tumor of brain	"	28	479.0	419.0	86.0
8	Pluriglandular insufficiency	Female	43	283.0	309.0	40.7
9	Cushing's syndrome	"	26	211.5	204.9	—
10	Syphilis of spinal cord	"	61	194.4	172.8	70.2
11	Hemolytic jaundice	"	33	209.0	197.6	43.6
12	Subacute bacterial endocarditis	Male	45	194.1	188.1	28.8
13	Congestive heart failure	"	54	341.0	249.1	71.3
14	Myocardial infarction	"	28	364.5	235.9	46.1

* ChE activity is expressed with CO_2 cubic mm. per 1 hour per brain tissue of 5 mg. wet weight.

TABLE III

Comparison between Brain ChE of Liver Patients and that of Controls

Part of the brain		No. of cases	Average value	95% confidence limits for the population mean
Putamen	Liver disease	13	228.9	$186.7 \leqq m \leqq 271.0$
	Control disease	14	254.9	$203.8 \leqq m \leqq 306.1$
Caudate nucleus	Liver disease	14	230.5	$170.0 \leqq m \leqq 291.0$
	Control disease	13	214.1	$148.7 \leqq m \leqq 279.4$
Pallidum	Liver disease	11	48.5	$22.8 \leqq m \leqq 74.2$
	Control disease	12	41.3	$26.2 \leqq m \leqq 56.4$

centration of ChE was measured with Warburg's apparatus, using the method of Ammon, as has been reported in our previous paper.[1] The ChE value was expressed by CO_2 cubic mm. liberated from Ringer's solution per hour per tissue of 5 mg. wet weight.

Results

ChE values of basal ganglia in patients of liver diseases (group 1) shown in Table I, and those in patients of control diseases (group 2) in Table II. Average values are indicated in Table III. It may be clearly seen that there is no appreciable difference between the general values of ChE in basal ganglia of groups 1 and 2. Although individual variation within a certain range is seen in the ChE value of corresponding parts of these brains, nothing seems to indicate the existence of a fixed and invariable influence of a disease on ChE content of basal ganglia.

Discussion

On the basis of experiments on rats, Mendel and others[5] stated that the ChE content of the brain and muscle showed no change by such treatments as thyroidectomy, starvation, and tumor implantation, though these had marked influence on the serum ChE level. Reports on similar observations on the human subjects apparently are not to be found. Whereas Nachmansohn[3] and Birkhäuser[4] investigated the distribution of ChE in the human brain, the latter reporting the values of 28 cases, only one instance of liver cirrhosis and two of gastrointestinal cancer were included in this group. In consideration of the findings mentioned in the introductory paragraph of the present report, we felt the necessity of studying further cases of liver cirrhosis. We investigated whether liver disturbance had any influnce on the ChE concentration of basal ganglia by using material from 28 cases, of which 14 were cases of liver disease. The result proved to be completely negative. The unknown factor which

connects liver and basal ganglia, noted in Wilson's disease, could not be detected in our study of ChE changes in ordinary liver diseases. We have had no occasion of directly studying the fresh sample of brain of Wilson's disease, as this is rarely obtained in our country. It is quite possible that in Wilson's disease high concentration of ChE in basal ganglia might play a significant part, since the disturbance of copper metabolism has been confirmed in this disease, while inhibition of ChE by copper is an observed fact. Studies on the pathogenesis of Wilson's disease are in progress on the basis of these considerations.

Summary

1. In order to study the influence of liver disease on ChE concentration of basal ganglia, 14 cases of liver diseases and 14 cases of control patients were investigated.

2. ChE values of putamen, caudate nucleus, and pallidum were measured and compared between these two groups.

3. No decrease of ChE in basal ganglia was confirmed in the group of patients of liver diseases. ChE of basal ganglia appears to remain unchanged in the ordinary liver diseases.

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Respiratory Arrhythmia and Change in Cardiac Rate in Hypertensive Patients with Change of Posture

By

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Many studies of changes in blood pressure and cardiac rhythm on postural changes have been reported. All of the authors, Guy (1900),[1] Stephens (1904),[2] Sewall (1916),[3] Henderson & Haggard (1918),[4] Ellis (1921),[5] Luton (1943),[6] Matsuda and Suzuki (1952)[7] obtained essentially the same results that cardiac rhythm was more frequent in standing position, and less frequent in recumbent than in sitting, although experimental conditions were not always the same.

Recently, this problem was studied by direct recording of intra-arterial blood pressure: Robert, Arnold & Johnson (1948)[8] suggested that change in cardiac rhythm in postural changes was brought about by the reflex mechanisms regulating blood pressure, and they interpreted the mechanism as a "breaking action."

Hering (1927)[9] who produced the hypertension experimentally in animal by cutting off the buffer nerves for the blood pressure regarded the role of the buffer nerves as of great significance in the pathogenesis of hypertension. Tanemura (1949)[10] reported that respiratory arrhythmia disappeared or became diminished considerably when the carotid bodies were extirpated in asthma patients and that the usual changes in cardiac rhythm due to postural changes became also much reduced.

We observed respiratory arrhythmia and changes in cardiac rhythm in hypertensive patients in detail by Matsuda's "*Cardiotachograph*" (1948),[11] a special apparatus which records automatically and continuously all changes of instantaneous cardiac rhythm, and compared them with those in normal subjects.

Experimental

Method

Thirty hypertensive patients, whose age ranged from 27 to 74 years, and nineteen normal subjects free of any sign of cardiovascular abnormality, aged from 21 to 61 years, were studied in this experiment. The subjects were made to alter their positions passively at a constant and moderate

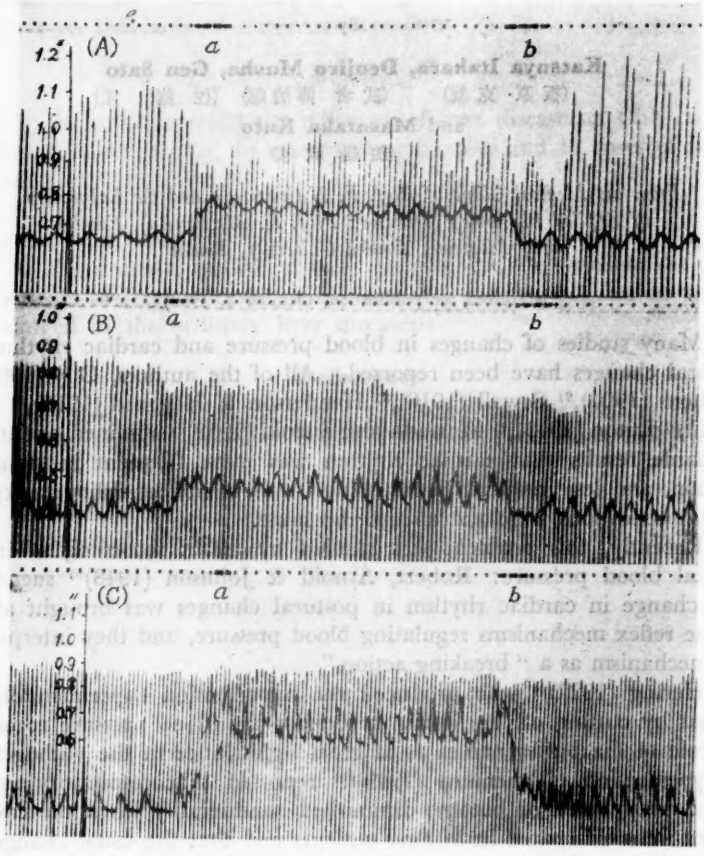

Fig. 1. Cardiotachogram of A) normal subject, B) benign and C) malignant hypertensive patient. Effect of change in posture. Upper: signal and time mark in two-second intervals. Middle: cardiotachogram, puls interval in second can be read in the scale at left. Lower: respiration, inspiration upward.

Subjects were made to alter their positions passively from horizontal to sitting at *a* and were returned to the original position at *b*.

speed from horizontal to sitting position after they had resumed recumbent posture for 20 minutes, and were kept in this position for 2 minutes. Then the subjects were returned to the horizontal position. Throughout the procedures the subjects were securely supported to exclude any active muscular activity to maintain their positions. Following these experiments the subjects changed actively the position from sitting to standing, after they had been at rest in sitting for 20 minutes; this position was maintained for 2 minutes, and then the subjects were returned to the previous sitting position again.

Cardiotachograms were taken continuously for 6 minutes throughout the postural changes (before, during, and following the postural changes respectively, each for 2 minutes).

Bipolar chest lead was used in order to avoid the disturbance which might occur in limb lead on postural changes; always the respiration was recorded simultaneously.[12]

Samples of such cardiotachograms are represented in Fig. 1.

The value of respiratory arrhythmia was measured in cardiotachograms as the difference between cardiac intervals in inspiratory and expiratory phases of respiration.[7] The value of respiratory arrhythmia of one subject expressed in this communication is the average of 20 values calculated as above. Average cardiac interval is the average of as many cardiac intervals as we can count in the cardiotachogram.

Results

1) Respiratory arrhythmia

Respiratory arrhythmia is generally in close connection with cardiac interval; the greater the latter is, the greater the former is.[7] The relations between the respiratory arrhythmia and the cardiac interval in hypertensive patients and normal subjects are illustrated in Fig. 2.

There is no prominent difference between hypertensive patients and normal subjects in the average cardiac rate, that is, the hypertensive patients have neither remarkable bradycardia nor remarkable tachycardia. As to the respiratory arrhythmia, however, there was noticed distinct difference as is shown by the frequency distribution of the values of respiratory arrhythmia in Fig. 3. The respiratory arrhythmia is smaller in hypertensive patients than in normal subjects (see Fig. 1.). It might be considered that the decrease in respiratory arrhythmia in hypertensive patients is rather more apparent than real because hypertension is more prevalent in aged individuals, in whom the respiratory arrhythmia is generally less marked than in younger individuals. Fig. 4 shows the respiratory arrhythmia of normal as well as of hypertensive patients

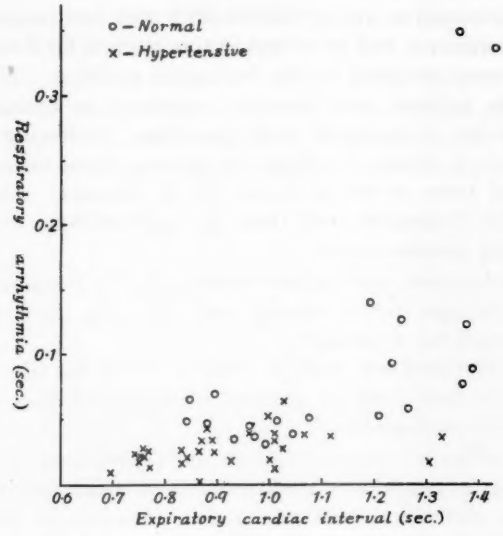

Fig. 2. Relation between expiratory cardiac interval and respiratory arrhythmia.

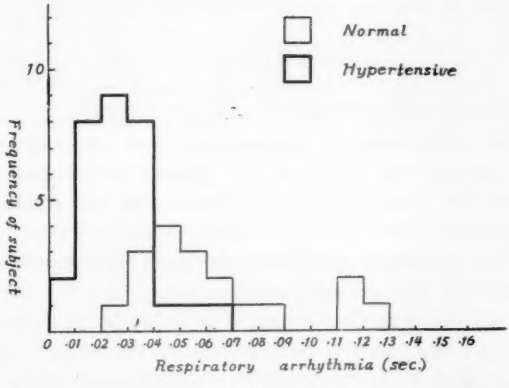

Fig. 3. Frequency distribution of the values of respiratory arrhythmia.

plotted against the ages of individuals. It can be realized from this figure that respiratory arrhythmia is smaller in hypertensive patients than in normal subjects, even if we take the influence of age into consideration.

2) Change in cardiac rhythm due to postural changes

The cardiac rate increases when position is changed from recumbent

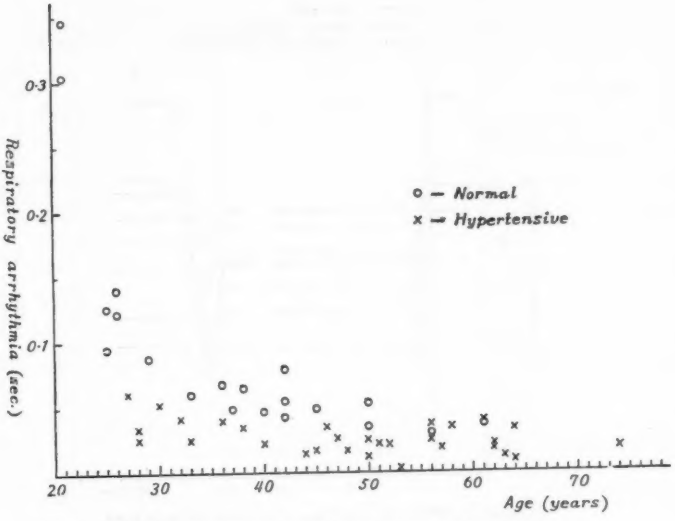

Fig. 4. Relation between age and respiratory arrhythmia.

to sitting and from sitting to standing postition, namely, cardiac interval becomes smaller respectively.

Fig. 5 (a, b) illustrates diagramatically the course of change in cardiac rhythm when hypertensive patients and normal subjects change their positions. The rate of the increase of cardiac beats, that is, the rate of diminution of the average cardiac interval is smaller in hypertensive patients than in normal subjects. This tendency is particularly prominent in the patients of malignant hypertension. Curiously enough, some patients showed the paradoxical reaction in each postural change. As there were also noted some effects of age on the change in cardiac rhythm in positional alterations, we examined and illustrated the relation between age and the difference of average cardiac interval due to postural changes in Fig. 6 (a, b). From the figure we can see that in patients with essential hypertension, the rate of decrease of the average cardiac interval is smaller than in normal subjects beyond the effect of the ages of individuals.

This becomes even more clear when we examine the frequency distribution of the difference of average cardiac interval due to postural changes (Fig. 7). In hypertensive patients the values converge near the origin of coordinate when respiratory arrhythmia is plotted against the change with cardiac rhythm in postural changes (Fig. 8). In other words, both the respiratory arrhythmia and the change in cardiac rhythm in postural changes are unusually small in hypertensive patients than in

Fig. 5. a

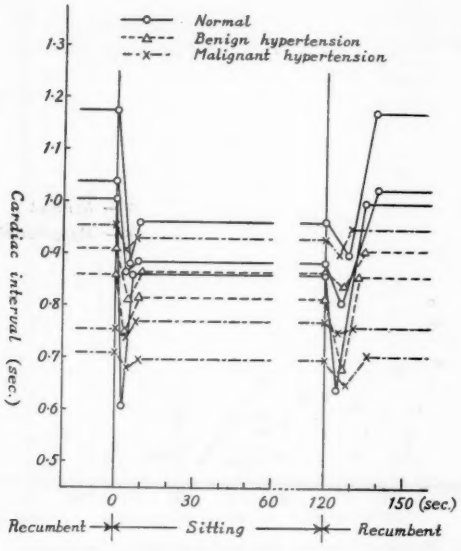

Fig. 5. b

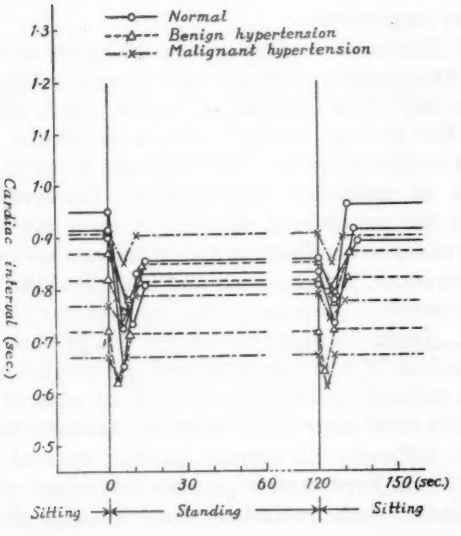

Fig. 5. Change in cardiac interval with postural changes (from recumbent to sitting position and return to the original position). Diagramatic representation. See explanation in text.

Fig. 6 a

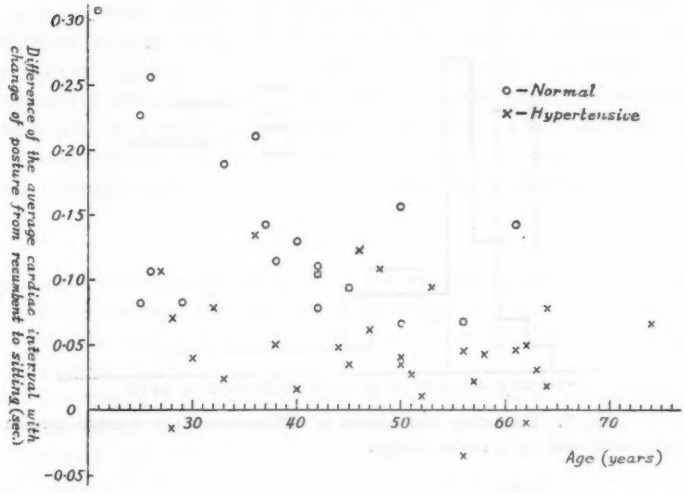

Fig. 6 b

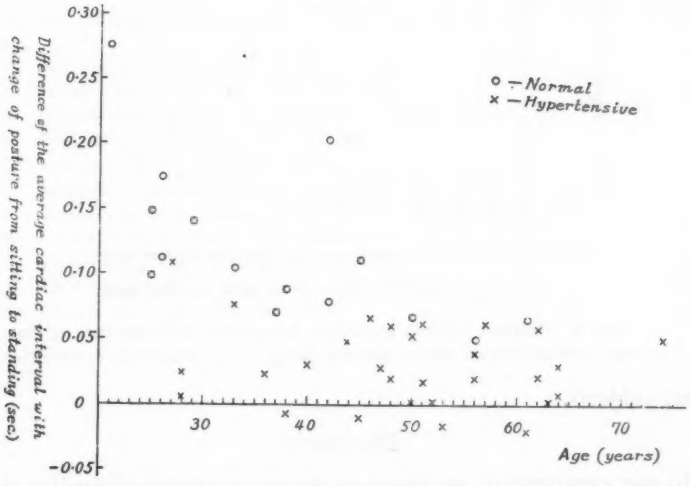

Fig. 6. Relation between age and difference of the average cardiac interval due to postural change. a: From recumbent to sitting, b: From sitting to standing.

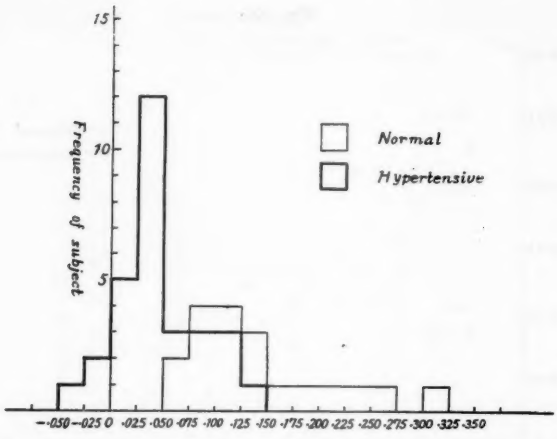

Fig. 7. Frequency distribution of difference of the average cardiac interval due to postural change.

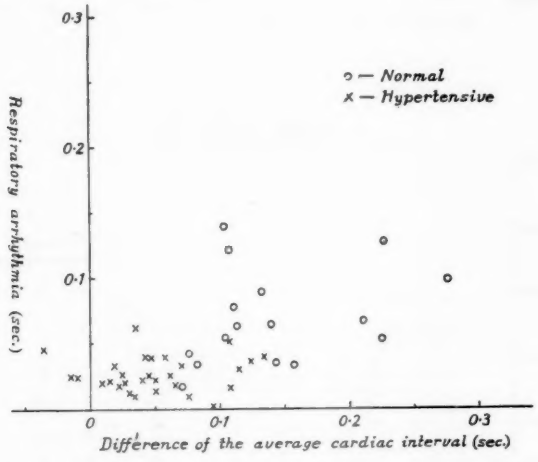

Fig. 8. Relation between respiratory arrhythmia and difference of the average cardiac interval due to postural change (from recumbent to sitting).

normal subjects.

Discussion

In our experiments, we found as the characteristic of cardiac rhythm in hypertensive patients 1) the decrease of respiratory arrhythmia and 2) diminution of the change in cardiac rhythm with change of position.

Though the physiological mechanism of such findings is not yet clear, it is not uncomprehensible that these characteristics are in close relation to the carotid sinus and/or aortic nerve reflex. The hypertension can be considered as due to the adaptation of the heart to maintain normal cardiac output against increased vascular resistance. It would nullify such adaptative action of heart if the buffer reflexes for the blood pressure work as active as normal under these conditions. The buffering action for the blood pressure of the carotid sinus and aortic nerves may be diminished through some unknown mechanism in hypertensive patients.

Conclusion

By means of " cardiotachograph " we studied respiratory arrhythmia and change in cardiac rhythm due to postural changes in thirty hypertensive patients, and the results were compared with those of nineteen normal subjects.

Our experiments revealed that both the respiratory arrhythmia and the change in cardiac rhythm with postural changes were smaller in hypertensive patients than in normal subjects. Such characteristic tendency was especially prominent in cases of malignant hypertension.

The authors wish to express their thanks to Prof. K. Matsuda and Prof. F. Nakazawa for their advice and encouragement.

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A Basic Antibiotic "Roseomycin" Produced by a Strain of *Streptomyces roseochromogenus*, No. 36

(Studies on the Antibiotic Substances from *Actinomyces*, 9th Report)

By

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Among about 300 active streptomyces,[1] collected chiefly in 1947 to 1949, so-called "streptothricin-like" substances producing strains were found in a relatively large number.

Further to differentiate these strains, secondary screening method was established, the idea and the method of which was already reported[2] and as the result streptothricin type 1 and 2 were classified. The former was of low potent, but low toxic and more diffusible in nature. Streptomyces No. 36 was a representative strain of this group. On the other hand, the latter was high active, but relatively high toxic and less diffusible. Streptomyces No. 259 was a representative strain of this group.

The present report deals with a new non-toxic, basic compound "roseomycin" produced by a strain of streptomyces No. 36, which is a representative one of the group of streptothricin type 1. The data was already reported in Japanese in 1950,[3] in detail.

Experimental

Taxonomic Study

As shown in Table I, the strain is closely resembling *Streptomyces roseochromogenus* (Jensen).

Activity of the Strain Tested with Cross-streaks

As shown in Table II, *B. anthracis* was more strongly inhibited than *B. subtilis* around the growing colony of streptomyces, while the cup assay of the filtrate and of the final antibiotic showed a quite contrary result. Cross-streaks against several antibiotic producing strains were also examined and the specific activity of this strain was proved as one kind of streptothricin.

TABLE I

The Taxonomic Characteristics of Streptomyces No. 0–36 compared with that of *S. roseochromogenus*[1)]

		Strain No. 0–36	*Streptomyces roseochromogenus*
Synthetic agar	growth	small, colorless	thin, spreading, colorless, penetrating deep into the medium
	aerial mycel.	hydrangea pink (5″f)-vinaceous (1″d), spreading, powdery, radiated	thin, pale brownish vinaceous (5‴f)
	soluble pigment	none	none
Glucose agar	growth	*snuff brown* (15″k)	*extensive, colorless*, spreading on and below the surface of the medium, edge entire
	aerial mycel.	pinkish vinaceous (5″d)	covering all surface, except wide margin; flesh-pink (5′f) color with white margin
	soluble pigment	none	none
Ca-malate agar	growth	yellowish olive (23″k)	spreading, colorless, growing deep into the medium
	aerial mycel.	white, radiated, spreading, powdery	white to rose colored
	soluble pigment	none	none
Starch agar	growth	colorless-dark plumbeous (49⁗i)-black plumbeous (49⁗k), small, raised	spreading, colorless
	aerial mycel.	white-cream color (19′f)-vinaceous buff (17‴d), powdery	white cottony tufts all over surface, with shade of pink
	soluble pigment	none	
	enzymatic zone	7.6 mm. wide	6–7 mm. wide
	diastatic action	very strong, all starch used up in 20 days, at 37°C	very good, all starch used up in 14 days from a 1% solution
Nutrient agar	growth	small, colorless-cream buff	white, later turning yellowish
	aerial mocel.	none	none
	soluble pigment	*faint yellowish brown*	*none*
Gelatin, 18°C	growth	cream colored colonies	small, cream-colored colonies, sinking into the medium
	aerial mycel.	none or thin white	none or thin white
	soluble pigment	brown	brown, spreading into the unliquified portion
	liquefaction	*rapid*	*slow*
Potato plug	growth	cream-buff (19″d)-saccardo's umber (17″k)	much winkled, brownish
	aerial mycel.	none	none
	color of plug	clove brown (17″m)	brown

Litmus milk, 37°C	growth (26°C)	water-green pellicle	brownish ring on surface in contact with glass
	coagulation	2–3 days	none
	peptonization	8–10 days	10–15 days
	change of reaction	pH 7.8	strongly alkaline
Glucose broth	growth	flakes on bottom and ring on surface	flakes on bottom, creamy ring on surface
	aerial mycel.	scant white ring	none
	soluble pigment	faint brown	brown
Blood agar	growth	small, wrinkled, chamois (19″b)	
	aerial mycel.	none	
	soluble pigment	none	
	hemolysis	none	
Peptone water	growth	cream-buff ring and flakes on bottom	
	aerial mycel.	none	
	soluble pigment	golden	
Nitrate reduction		positive	positive
Tyrosinase		negative	negative
Spirals		open type spirals from one branching hyphae	numerous open and closed spirals produced on the different synthetic media
Conidia		synthetic agar, more or less cylindrical, 0.8–1.0×1.0–1.3μ	on synthetic agar, oval, 1.0–1.2×1.5–3.0μ

1) Breed, K. S. *et al.*: "Bergey's manual of determinative bacteriology" 6 ed., The Williams and Wilkins Company, Baltimore, 1948. 2) It is noteworthy that properties in italic are different from each other.

Cultural Condition for Production of Antibiotics

After the examination of many kinds of media in both surface and shaking culture methods, shaking culture with modified Vander Brook's medium was found to be most suitable to obtain a potent culture filtrate. As the result the production medium selected is as described below: 1% glucose, 1% peptone, 0.25% ammonium sulfate, 0.025% magnesium sulfate, 0.1% dipotassium phosphate, 0.5% sodium chloride, 0.35% calcium carbonate were mixed in distilled water and adjusted to pH 7.0.

1.5 to 2 mg. per cc. of hydrochloride was produced under above condition after 3–4 days at pH 7.6 to 7.8.

Purification Procedure

The purification method was nearly the same, with that of strepto-

TABLE II

Cross-Streaks of the Strain No. 36 Against Bacteria and Antagonistic Streptomyces

Test organisms	Inhibition zones (in mm.)
Ps. aeruginosa	0
Alk. faecalis	8.0
E. coli	9.0
E. coli (Streptomycin resistant)	9.0
Kl. pneumoniae	6.7
Sh. sonnei	11.1
Sta. aureus (F.D.A. 209–P)	11.0
B. subtilis	4.5
B. anthracis	9.2
My. smegmatis	10.7
My. avium	10.9
My. phlei	13.2
Roseomycin prod. strain (No. 36)	0
Streptomycin prod. strain (B_3)	24.2
Chloromycetin prod. strain (Hosoya)	13.0
Streptothricin type 2 prod. strain (259)	6.2
Streptothricin type 1 prod. strain (93)	0
Streptothricin type 1 prod. strain (98)	0

mycin and streptothricin.

The potency of culture filtrate reached 1,600 *Sta. aureus* dil. unit and 320 *E. coli* dil. unit after 4–5 days in surface, and 3,200 *Sta. aureus* unit and 640 *E. coli* unit after 3–4 days in shaking culture condition.

The culture filtrate was combined and adjusted to pH 3.5. The mycelium and precipitate was removed by filtration and further by treatment with 0.3% charcoal, stirring for 15 minutes at acidic pH (pH 2.0), some impurities were removed and decolorized. Clarified filtrate, thus obtained, was further adsorbed with 1 to 1.5% charcoal at neutral pH, and the charcoal was washed with water and neutral 10% acetone several times. Then the carbon cake was eluted with acidic 10% acetone (pH 2.0, 1/10 volume of the filtrate) 2 times. Almost all the activity was recovered. These eluates were combined and adjusted to pH 5.0 with NaOH and occurring flocculous precipitate was removed. After the concentration in vacuo to dryness the agent was redissolved in N/10 anhydrous HCl-methanol in order to remove inorganic salt. Further the active agent was precipitated from the methanol solution by acetone. The final procedure was repeated 2 times and the crude hydrochloride was obtained in a white amorphous powder. These hydrochloride pos-

sessed 85 to 92% purity when compared with standard salt.

Crystalline reineckate (d.p. 114°C) and crystalline helianthate (d.d. 211–216°C) was induced from hydrochloride. The conversion of helianthate to hydrochloride was also examined, but the yield was not suitable for mass production.

Chromatographic separation was performed using Al_2O_3 (Japan Alum. Co. Ltd. A.P. 6) as adsorbent column according to Cartor[4] *et al.* The one example of the experiment was shown in Table III.

TABLE III

An Example of Chromatographic Purification

Fraction (No)	Volume (cc)	Potency (u/cc)	Color	Precipitate		Purification
				by 2N NaOH	by Acetone	
1	16	<1250	no	+	−	
2	34	<1250	no	+	−	
3	14	13000	pale yellow	##	##	
4	20	3800	no	++	++	From frac. 3 a
5	20	<1250	no	+	−	pure hydrochloride
6	40	1300	no	−	−	(purity 1.02) in
7	40	1640	slightly yellowish	−	−	amount of 161.3 mg was obtained
8	40	<1250	slightly yellowish	−	−	
9	40	2750	yellowish	−	−	

Direction: According to Carter *et al.*, the column was developed with 80% methanol. Lot. 7, 8 and 9 are the eluate from the Al_2O_3 after the current course, with acidic 80% methanol under stirring.

Material: 1.3 g. of crude hydrochloride (purity 0.82) was supplied and the yield in potency was 47%.

Color Reactions of Hydrochloride

Crude hydrochloride showed a slightly postitive biuret and Sakaguchi test, but purified one was negative in biuret, ninhydrin, xanthoproteine, Adams-Kiewitz, Lieberman, Neubauer, Sakaguchi and Maltol test. Elson-Morgan's glucosamine, Molisch' (violet), Tollens' naphthoresorcine (brown) and indol (brown) test was all positive. Also Fehling's solution was reduced. All above reactions were tested with the solution in concentration of 100 mg./40 cc. aqueous solution.

Antibacterial Activity of the Pure Hydrochloride

Antibacterial spectrum was compared with that of streptomycin both with diffusion and dilution method (Table IV and V). The activity of roseomycin against gram positive cocci was somewhat lower than strepto-

TABLE IV

Antibacterial Spectrum of Roseomycin Tested by Diffusion Method (Cup Assay) Compared with that of Streptomycin

Test Organisms	HCl-Salt of Antibiotics (1 mg/cc)	
	Roseomycin[1]	Streptomycin[1]
Gram Positive Organisms		
Staphy. aureus (Terashima)	14.7	19.4
Staphy. aureus (F.D.A. 209–P)	13.8	19.3
B. agri[2]	0	0
B. anthracis	4.4	22.6
B. subtilis	15.2	15.6
Gram Negative Organisms		
E. coli	14.9	18.1
SM. resist. *E. coli*[3]	15.0	0
RM. resist. *E. coli*[3]	0	3.7
259 resist. *E. coli*[3]	0	9.6
20 resist. *E. coli*[3]	0	8.0
120 resist. *E. coli*[3]	0	11.0
Alkalig. faecalis	11.0	11.0
S. typhosa	11.5	10.4
Sh. sonnei	16.9	16.4
Kl. pneumoniae	15.7	10.3
Ps. aeruginosa	0	4.0
Acid Fast Bacteria		
Myco. smegmatis	12.0	19.5
Myco. phlei	11.0	13.0
Myco. avium	10.1	10.2
Myco. Sp. (Clegg)	10.4	10.8
Myco. sp.	6.7	10.5

1) All inhibition zones were measured in mm. 2) *B. agri* (Hosoya) resembled the Bodenheimer's organisms, being resistant to streptomycin and susceptible to streptothricin like substances. 3) All these mean *E. coli* resistant to streptomycin, roseomycin and streptothricin like basic substances, No. 259, No. 20 and No. 120, isolated and crystallized in our laboratory.

mycin, while against gram negative and non-pathogenic acid-fast organisms, almost equivalent activities were proved. Moreover it is noteworthy to describe that roseomycin was more inhibitory to *Vibrio comma* than streptomycin, while against *Myt. tuberculosis* the latter was much more effective than the former.

B. agri was not inhibited both with streptomycin and roseomycin, but inhibited by streptothricin type 2. Inconformities of the antibacterial spectra between that of growing streptomyces and of pure hydrochloride was observed against *B. subtilis* and *B. anthracis*.

Diffusion Curve

Diffusibility of streptomycin and roseomycin was compared[1] with

TABLE V

Antibitoc Spectrum Tested by Dilution Method

Test Organisms	Roseomycin[1]	Streptomycin[1]
Staphylococcus aureus (Terashima)	1.0	0.1
Staphylococcus aureus (F.D.A. 209–P)	1.0	0.1
Streptococcus hæmolyticus	25.0	1.6
Diplococcus pneumoniæ[2] (I)	12.5	3.0
Neisseria meningitidis[2]	25.0	3.0
Corynebacterium diphtheriæ[2] (PD)	10.0	3.0
Bacillus subtilis (NRRL 588)	25.0	2.5
Bacillus anthracis (Umezawa)	50.0	0.3
Escherichia coli	5.0	0.6
Streptomycin-resistant E. coli	3.0	>200.0
Alcaligenes fæcalis	5.0	5.0
Salmonella typhosa	1.8	2.5
Salmonella paratyphi	1.2	1.6
Salmonella schottmuelleri	1.6	1.2
Shigella dysenteriæ	1.0	0.5
Shigella sonnei	1.2	0.1
Klebsiella pneumoniæ	2.5	2.5
Pseudomonas aeruginosa	50.0	2.5
Hemophilus pertussis[3]	37.5	3.0
Pasteurella tularensis[4]	12.0	1.6
Vibrio comma	1.0	>100.0
Mycobacterium tuberculosis var. hominis[5]	20.0	1.2
Mycobacterium avium[6]	1.6	2.5
Mycobacterium smegmatis[6]	0.8	0.4
Mycobacterium phlei[6]	5.0	2.5
Clostridium perfringens[7]	>100.0	>100.0
Clostridium tetani[7]	>100.0	>100.0
Clostridium botulinum[7]	50.0	50.0

1) Shows minimum inhibitory amount of HCl-salt in microgram. 2) Nutrient broth containing 1% glucose used. 3) 10% blood agar used. 4) Nutrient broth containing 0.1% cystine used. 5) Dubos' medium used. 6) Nutrient broth containing 4% glycerol used. 7) Tarozzi's liver liver broth used. Other organisms were tested in less nutrient broth (pH 7.5).

superposing method (one dimensional diffusion assay) as shown in Table VI and Fig. 1. The activity ratio of streptomycin and roseomycin against *Sta. aureus* with dilution method is 10:1, so a good diffusibility of roseomycin will be anticipated from the inhibition curve.

Acquired Resistance against Roseomycin

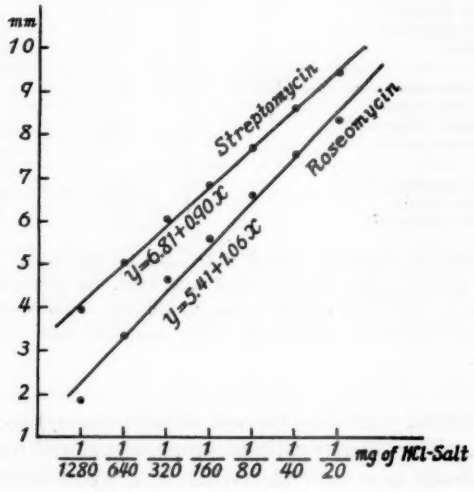
E. coli can be induced into a resistant strain by transfers in roseomycin containing culture medium. i.e. the susceptibility of this organism, which was originally 1/60 μg. was shifted to 1/2 μg. (80 fold), after the transfers in roseomycin containing broth, and further transfer could not increase the grade of resistance.

TABLE VI

Diffusibility of Roseomycin Compared with that of Streptomycin. (Assayed by Superposing Method, Tested with *Staphylococcus aureus* (Terashima))

HCl-salt (mg.)	Roseomycin					Streptomycin				
	Inhibition zones (mm.)				Aver.	Inhibition zones (mm.)				Aver.
	1	2	3	4		1	2	3	4	
1/20	8.3	8.3	8.3	8.4	8.325	9.4	9.5	9.4	9.4	9.425
1/40	7.5	7.5	7.6	7.5	7.525	8.7	8.6	8.6	8.6	8.625
1/80	6.6	6.6	6.5	6.7	6.600	7.7	7.7	7.6	7.7	7.700
1/160	5.6	5.5	5.6	5.6	5.575	6.8	6.9	6.9	6.9	6.875
1/320	4.6	4.6	4.7	4.6	4.625	6.0	6.1	6.0	6.0	6.025
1/640	3.2	3.4	3.4	3.4	3.350	5.0	5.0	5.0	5.1	5.025
1/1280	1.8	1.8	1.9	2.0	1.875	4.0	4.0	3.9	4.0	3.975

Fig. I. Diffusibility of Roseomycin Compared with that of Streptomycin.



Stability

Generally speaking, roseomycin was stable at acidic pH. Boiling at 100°C for 20 min. reduced the activity as follows: At pH 2.0–90%, pH 5.0–92%, pH 7.0–71% and pH 9.0–50%.

Toxicity

20 mg. of hydrochloride were injected both intravenously and in-

tramuscularly to mice weighing 15 to 20 g. No toxic signs were observed. Further 40 mg. were injected by both routes, and only 1 mouse died after 18 hours. But no hemorrhage, as usually found with streptothricin type 2, was observed. Amniotic injection of 0.5 mg. into fertile eggs was also non-toxic.

In vivo Activity

Against typhoid, cholera and tularaemia infection in mice, the activity was proved and will be reported later.

Against *Rickettsia prowazeki* in both egg and guinea pig experiment it was proved to be non effective.

Summary

A streptothricin-like substance "roseomycin" was purified from culture filtrate of a strain of *Str. roseochromogenus*. Crystalline reineckate and helianthate was induced. Pure hydrochloride, positive in glucosamin test, was active against gram negative organisms in a similar degree as streptomycin. The toxicity seems to be very low. A high activity against *V. comma* and against streptomycin resistant organisms seems to be so favourable with this antibiotic.

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A Basic Antibiotic Produced by a Strain of *Streptomyces roseochromogenus*, No. 24

(Studies on the Antibiotic Substances from *Actionomyces*, 10th Report)

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My collaborators have already reported on the systematic studies of the antibacterial streptomyces,[1-2] and Ishida isolated "roseomycin"[3] from a strain of *Streptomyces roseochromogenus*.

A strain of streptomyces No. 24, which seems also to be resembling to *Sreptomyces roseochromogenus* (Jensen), showed an interesting antibacterial spectrum. As usually known, streptomycin producing strains inhibit *B. anthracis* (Umezawa strain) more strongly than *B. subtilis* (NRRL 588), while strains producing streptothricin-like substances inhibit *B. subtilis* more strongly than *B. anthracis*, when tested by cross-streaks. The strain No. 24 which inhibits *B. anthracis* more strongly than *B. subtilis* when tested by the same method, appeared to be a streptomycin procuding one. This property, however, was lost if it was tested by the cup assay using its culture filtrate (the secondary screening test). The imconformity of antibiotic spectrum shown by cross-streaks (the primary screening) with that of the cup assay tested with culture filtrate has also been found in many other cases, and was presumed to be due to another antibiotic substance, which did not appear in the culture filtrate.

The present paper is to report on the purification procedure of an antibiotic produced by the strain No. 24, and on the changes of antibacterial spectrum of growing cultures, of the culture filtrates and of the final antibiotics.

Experimental

Activity of the strain. As shown in Table I., the strain is closely resembling *Streptomyces roseochromogenus* (Jensen).

As shown in Table II, it was active against gram positive, negative

TABLE I

Mycological Properties of Strain No. 24

	Media	Growth	Aerial mycelium	Soluble pigment	Biochemical features
Cultural characteristics	Synthetic agar	Colorless	Pale brownish vinaceous (Rdg. 5‴f) colored	None	
	Glucose agar	Colorless	Fresh pink at first, later becoming light brownish vinaceous colored	None	
	Nutrient agar	Brownish	None	Brown	
	Starch agar	Colorless	At first white, later light brownish vinaceous (Rdg. 5‴ d) colored	None	
	Potato plug	Brown	Grayish brown	Brown	
	Gelatin, 18°	Cream color (Rdg. 19′f)	White	Brown	Liquefaction: slow
	Glucose broth	Yellowish brown	None	Brown	
	Blood serum, 37°	Gray	None	Faint brown	Liquefaction: slow
Morphology	Spirals	No spirals formed on synthetic agar			
	Conidia	Oval shaped, 1.5×2.0 microns			

TABLE II

Activity of the Strain No. 24 Tested by Cross-Streaks

Test bacteria	Inhibition zones (in mm.)
Ps. eruginosa	0
Alk. fæcalis	10.7
E. coli	9.8
E. coli (SM resistant)	8.0
Kl. pneumoniæ	6.1
Sh. sonnei	10.5
Sta. aureus	11.7
B. subtilis (NRRL 588)	10.0
B. anthracis (Umezawa)	11.0
Myco. smegmatis	9.0
Myco. avium	11.0
Myco. phlei	9.5

Streptomyces No. 24 was cultivated in the center of a plate at 27°C for 3–4 days and the test organisms were steaked radially around the colony of streptomyces and incubated at 37°C, for 24 hours. Inhibition zones, thus produced were measured in mm.

and acid-fast organisms. *B. anthracis* is more strongly inhibited than *B. subtilis*.

TABLE III

Activity of the Filtrate in Cup Assay

Test organisms	Inhibition zones (in mm.)
Sta. aureus (Terashima)	10.0
Sta. aureus (F.D.A. 209–P)	8.6
E. coli	5.1
E. coli (SM resistant)	7.3
E. coli (roseomycin resistant)	6.1
B. agri	0
B. subtilis (NRRL 588)	7.7
B. anthracis	7.0

TABLE IV

Stability of the Filtrate on Boiling

pH	Boiling at 100°C for				Non treated
	5 min.	15 min.	30 min.	60 min.	
5.0	2560*	2560	2560	1280	2560
7.0	1280	1280	640	160	
8.0	1280	1280	320	40	

* Dilution unit using *Sta. aureus* as test organism.

TABLE V

Activity of Crude Hydrochloride of No. 24 in Cup Assay

Test organisms	Inhibition zones (in mm.)	
	Streptomycin-HCl	No. 24–HCl
E. coli	18.1	10.9
E. coli (SMR)	0	10.0
E. coli (RMR)	3.7	0
Alk. fæcalis	11.0	4.8
S. typhosa	10.4	10.7
Sh. sonnei	16.4	12.4
Kl. pneumoniæ	10.3	8.0
Ps. æruginosa	4.0	0
Sta. aureus (Terashima)	19.4	11.4
Sta. aureus (F.D.A. 209–P)	19.3	10.8
B. agri	0	0
B. anthracis	22.6	6.4
B. subtilis	15.6	15.4
Myco. smegmatis	19.5	12.0
Myco. phlei	13.0	11.4
Myco. avium	10.2	6.0
Myco. sp. (Klegg)	10.8	7.9
Myco. sp.	10.5	8.0

Concentration of test solutions: 1 mg./ml. Culture media: nutrient agar for Gram negative and positive bacteria and 4% glycerol nutrient agar for acid-fast bacteria. The assay was performed being compared with streptomycin.

TABLE VI

An Example of Chromatographic Purification

Fraction (No.)	Eluates					Hydrochloride			
	Volume (cc.)	Precipitate with 2 N NaOH	Precipitate with acetoue	Sakaguchi test	Activity (μg/cc.)	Yield (mg.)	Activity (dil. unit/g)		d. p. of helianthate
							Sta. aureus	E. coli	
1	30	−	−	##	4,000	157	200,000	200,000	270°C–274°C
2	20	##	−	++	3,800	263	800,000	800,000	276°C–280°C
3	40	##	++	+	85,000	495	4,000,000	800,000	280°C
4	40	++	##	+	55,000				
5	50	+	++	−	3,400	522.5	200,000	200,000	
6	50	−	+	−	560				
7	10	−	±	−					
8	30	−	−	−					
9	20	−	−	−	900	273.2	100,000	5,000	260°C–274°C
10	20	−	−	−	800				
11	20	−	−	−	600				
12	20	−	−	−	300				

Direction: According to Ishida, the column was developed with 80% methand. Lot 9, 10, 11 and 12 are the eluates from the Al_2O_3 with acidic 80% methanol with stirring after the current procedure.

Material: 10 g. of curde hydrochloride (200,000 *Sta. aureus* dil. unit./g.) was supplied and the yield in potency was 68%. Other techniques used in the manipulation were conducted as similarly as those employed by Cartor *et al.*

TABLE VII

Activity of Purified No. "24" HCl-salt

Test-bacteria	Diffusion assay (mg/cc)		Dilution assay	
	No. 24 HCl salt	Strepto-mycin	No. 24 HCl salt	Strepto-mycin
Sta. aureus (Terashima)	17.6	20.9	4,000,000	8,000,000
Sta. aureus (F.D.A. 209–P)			4,000,000	8,000,000
B. subtilis	19.1	18.8	800,000	800,000
B. antheracis	10.5	23.1	640,000	2,000,000
E. coli	15.8	17.4	800,000	800,000
E. coli (SM resistant)	17.4	0	800,000	5,000
Alk. fæcalis	13.7	12.4	800,000	400,000
Sh. sonnei	19.9	19.9	2,000,000	8,000,000
Kl. pneumoniæ	15.9	14.9	400,000	400,000
Ps. æruginosa	4.1	10.9	500,000	2,000,000
Myco. smegmatis	14.5	21.0	100,000	800,000
Myco. phlei	9.6	19.3	100,000	400,000
Myco. avium	17.9	17.9	100,000	100,000
Myco. tuberculosis (Dubos' Med.)			10,000	800,000

The assay was performed in comparison with streptomycin.

Cultural condition for production. Many kinds of media, both in surface and on shaking condition were tested at 26°–28°C. In surface culture glucose was proved to be the most suitable as carbon source and the more nitrogen sources were required for higher production. In shaking culture (6 cm. in amplitude, 110 r.p.m.) Waksman's medium was chiefly examined and also in this condition more nitrogen sources were required to obtain potent broth. For mass production surface culture using a medium has been selected, which was composed of 1% meat extr., 1% peptone, 0.5% NaCl and 1% glucose.

Some biological properties of the filtrates. Antibacterial spectrums was shown in Table III. Contrary to the result of cross-streaks, *B. subtilis* is more strongly inhibited than *B. anthracis*, and the filtrate was proved to be active against "roseomycin" resistant organisms too. As shown in Table IV, the activity of filtrate was confirmed to be most stable at pH 5.0. Filtrability with Seitz EK-filter at pH 7.8 has been also proved. A higher activity is shown when tested in alkaline broth (pH 8.0).

Purification procedure The purification method was nearly the same, with that reported previously.[3] The potency of culture filtrate reached 1280–2560 *Staphylococcus* dil. unit and 80–160 *E. coli* unit after the incubation for 7–8 days at pH 7.6–7.8.

The culture filtrates were combined and adjusted to pH 3.5. The mycelium and precipitate were removed by filtration and further the filtrate was decolorized by treatment with 0.5% charcoal for 30 minutes at acidic pH (pH 2.0). Clarified filtrate, thus obtained, was further adsorbed with 2% charcoal at neutral pH, and the charcoal was washed with water and neutral 10% acetone several times. Then the carbon cake was eluted with acidic 10% acetone (pH 2.0, 1/10 volume of the filtrate) 2 times. Almost all the activity was recovered. These eluates were combined and adjusted to pH 5.5 with NaOH and occurring flocculous precipitate was removed.

After the concentration of the supernate in vacuo to dryness, the residue was redissolved in anhydrous N/10 HCl-methanol in order to remove inorganic salt. The active agent was precipitated from the methanol solution with acetone. The final procedure was repeated and the crude hydrochloride (200,000 *Sta. aureus* dil. unit/g.) was obtained in a white powder with 50% yield, the activity of which was shown in Table V in comparison with streptomycin. This hydrochloride was induced to helianthate (d.p. 252–264°C) and recrystallized with 50% methanol (d.p. 275°). The final recrystallization was made with 70% methanol (d.p. 275°~280°). This helianthate was converted to hydrochloride with acidic methanol, the activity of which was confirmed to be 400,000 u/g. twice of the starting material.

Other method of purification was a chromatographic procedure. One example was shown in Table VI. Pure hydrochloride obtained from Frac. (3, 4) possessed a activity of 4,000,000 *Sta. aureus* unit/g, 10 fold of the starting material. The antibacterial spectrum was compared with that of streptomycin and shown in Table VII.

Chemical and biological properties of HCl-salt. Decomposition point of reinduced crystalline helianthate was 275°–285°C and that of crystalline reineckate 185–201°C.

The preparation was negative in biuret, ninhydrin, xanthoprotein, Adams-Kiewitz, Lieberman, and Neubauer test, and positive in Sakaguchi (pale red), Elson-Morgan's, Molisch's, Tollens', and Indol test, using 100 mg./40 cc. aqueous solution.

Though an intramuscular injection of 20 mg. of this salt was not fatal to mice, they died by an intravenous injection of 5 mg. after 3 days and showed some hemorrhagic changes in the liver and lungs. This product was inactive against roseomycin resistant organisms, contrary to the result obtained with culture filtrate.

Summary

1) A streptothricin-like substance, belonging to streptothricin type I was isolated from the culture filtrate of *Str. roseochromogenus* No. 24.

2) The in vitro activity was nearly the same with that of streptomycin, but 5 mg. injected intravenously into mice, represented a delayed toxicity.

3) Though the crystalline helianthate and reineckate of this salt differ from those of streptothricin type I (roseomycin) in their decomposition points, these compounds seem to be quite similar to the latter in other respects.

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Three Basic Antibiotics, Streptomycin, Roseomycin and No. 259 in Experimental Typhoid Infection

(Studies on the Antibiotic Substances from *Actinomyces*, 11th Report)

By

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There are many cases of typhoid fever in our country, but no effective chemotherapeutic agents have been devised yet to treat this infection. The antibiotics obtained from streptomyces, such as streptomycin,[1)2)] chloramphenicol,[3)] aureomycin and terramycin,[4)] seem to be very beneficial against typhoid infections.

During the systematic study of the antagonistic actinomyces in our laboratory, streptomycin and many streptothricin-like substances were isolated and crystallized.[5)] The present study deals with activities of streptomycin and two streptothricin-like substances against *Salmonella typhosa* both in vitro and in experimental infection. (The experiment conducted in 1949).

Experimental

Materials and Methods

1) Streptomycin used was HCl-calcium chloride double salt, Merck.

2) Roseomycin[6)] (No. 36 HCl-salt) was a representative substance of the so-called streptothricin type 1 (ST 1) of our laboratory, which was characterized in low toxicity, good diffusibility and low potency against gram positive organisms.

3) No. 259 HCl-salt[5)] was a representative substance of the so-called streptothricin type 2 (ST 2), and characterized in high potency, less diffusibility and relatively high toxicity.

In vitro activity was tested on one hand by serial dilution method with nutrient broth (1% meat ext., 1% peptone and 0.3% NaCl, pH 7.5). The total volume of test fluid divided into each tube was 5 cc. One drop (0.05 cc.) of 10 fold dilution of 24 hours broth culture of test

TABLE I

Activity Ratio of 3 Antibiotics against *E. coli* and *S. typhosa*.
(Dilution Unit)

Antibiotics	Test organisms		Activity ratio *S. typhosa* : *E. coli*
	E. coli (Umezawa strain)	*S. typhosa* (T-63 strain)	
a. Streptomycin	160*u/mg.	20 u/mg.	8/1
b. Roseomycin	16	20	1/1
c. No. 259 substance	80	200	1/2
Ratio (a : b : c)	10 : 1 : 5	1 : 1 : 10	

* Minimum amount required for complete inhibition

TABLE II

Dilution Unitage of Streptomycin and Roseomycin Against
6 Strains of *S. typhosa*

Strain tested	μg of the antibiotics contained in 1 cc. of broth										Control
	Streptomycin					Roseomycin					
	$\frac{1}{10}$	$\frac{1}{20}$	$\frac{1}{40}$	$\frac{1}{80}$	$\frac{1}{160}$	$\frac{1}{10}$	$\frac{1}{20}$	$\frac{1}{40}$	$\frac{1}{80}$	$\frac{1}{160}$	
No. 50	−	−	±	+	+	−	−	+	+	+	###
No. 53	−	−	++	++	++	−	−	+	+	++	###
No. 58	−	−	−	+	++	−	−	±	+	++	###
T-63 (U.S.A.)	−	−	+	+	+	−	−	±	+	+	++
No. 14*	−	−	±	+	++	−	−	±	+	++	++
Haryu-strain	−	−	+	++	++	−	−	±	±	+	++

###, ++, + = visible growth, showing its grade. ± = incomplete inhibition. − = complete inhibition. * Strains of *S. typhosa* preserved in the author's laboratory.

organisms, i.e *S. typhosa* and *E. coli*, were inoculated into each tube. Diffusion assay was conducted on the other according to the standard pulp disc assay of streptomycin.

In vivo activity was tested in mice weighing 15 to 23 g., and challenged with *S. typhosa* (T-63 strain), suspended in 5% mucin solution.

Results

As shown in Table I, activity ratio of 3 antibiotics i.e. streptomycin, roseomycin and No. 259 against *E. coli* was 10:1:5, while that against *S. typhosa* was 1:1:10. Although in vitro test, No. 259 seems to be most effective in such a manner, the preliminary test in vivo showed a contrary result. From this point of view, chiefly roseomycin and streptomycin

TABLE III

Efficiency of Streptomycin, Reseomycin, and No. 259 Substance in Protecting Mice against *S. typhosa*, Administered Intraperitoneally Immediately after Inoculation

Animal group	No. of mice	mg of organisms per 0.5 cc. in mucin solution injected intraperitoneally	Single dosage (mg.)	No. of mice that died on day										Death rate	Level of significance (%)
				1.	2.	3.	4.	5.	6.	7.	8.	9.	10.		
Control	5	10^{-4}		5										5/5	
	5	10^{-5}		4	1									5/5	
	5	10^{-6}		3	0	2								5/5	
	5	10^{-7}		1	0	1	0	0	0	0	0	0	0	2/5	
Streptomycin	5	10^{-6}	0.03	1	0	0	0	0	0	0	0	0	0	1/5	2.4
	5	10^{-6}	0.05	0	0	0	0	0	0	0	0	0	0	1/5	2.4
	5	10^{-6}	0.1	0	0	0	0	0	0	0	0	0	0	0/5	0.4
No. 259 substance	5	10^{-6}	0.06	1	0	0	0	0	0	0	0	1	0	2/5	8.3
	5	10^{-6}	0.1	0	0	1	0	0	0	0	0	0	0	1/5	2.4
	5	10^{-6}	0.2	0	1	0	0	0	0	0	0	0	0	1/5	2.4
Roseomycin	5	10^{-6}	0.075	0	1	0	0	0	0	0	0	0	0	1/5	2.4
	5	10^{-6}	0.125	0	0	0	0	0	0	0	0	0	0	0/5	0.4
	5	10^{-6}	0.25	0	0	0	0	0	0	0	0	0	0	0/5	0.4

TABLE IV

Efficiency of Streptomycin and Roseomycin in Protecting Mice against *S. typhosa**

Animal groups	No. of mice	Single dosage mg.	Administration method	No. of mice that died on day										Death rate	Level of significance (%)
				1.	2.	3.	4.	5.	6.	7.	8.	9.	10.		
Control	15			9	4	0	0	0	0	0	0	0	0	13/15	
Streptomycin group	10	0.05	Intraperitoneal	0	5	0	0	1	0	0	0	0	0	6/10	14.7
	5	0.2	Intramuscular	2	0	0	0	0	0	0	0	0	0	2/5	7.3
	5	0.2	Intravenous	1	1	0	0	0	0	0	0	0	0	2/5	7.3
	5	0.4	Intramuscular	0	0	0	0	0	0	0	0	0	0	0/5	0.14
Roseomycin group	10	0.05	Intraperitoneal	3	3	0	0	1	0	0	0	0	0	7/10	24.8
	5	0.2	Intramuscular	2	0	0	0	0	0	0	0	0	0	2/5	7.3
	5	0.2	Intravenous	1	0	1	0	0	0	0	0	0	0	2/5	7.3
	5	0.4	Intramuscular	0	0	0	9	0	0	0	0	0	0	0/5	0.14

* Strain: T-63 (U.S.A.)., Dose of organisms: 10^{-6}mg. in 0.5 cc. of 5% mucin solution. Inoculations were made intraperitoneally. Drugs were administered 1 hour after the infection.

were selected further to test on their activities in vitro against 6 strains of *S. typhosa*. Almost equivalent activity was indicated with both anti-

TABLE V

Diffusion Activity of 3 Antibiotics against *S. typhosa* T-63

μg of HCl salt	Streptomycin						Roseomycin						No. 259 substance					
	I	II	III	IV	V	Aver.	I	II	III	IV	V	Aver.	I	II	III	IV	V	Aver.
31	17.0	17.0	16.4	16.7	16.4	16.70	16.9	17.0	17.0	17.5	16.3	16.94	12.3	11.4	12.6	12.2	12.3	12.16
63	19.2	18.7	18.9	17.6	18.6	18.80	18.9	18.7	18.6	19.4	18.4	18.80	14.4	13.9	14.6	14.3	14.6	14.36
125	21.4	20.7	21.0	20.8	21.1	21.00	21.4	20.8	20.5	21.6	21.0	21.06	16.2	15.8	16.2	16.1	16.3	16.12
250	23.2	22.0	23.3	22.4	23.0	22.78	23.2	22.6	22.5	23.4	22.0	22.74	17.9	17.7	17.4	17.4	17.5	17.58
500	24.5	23.3	24.4	24.2	24.2	24.12	24.2	23.8	23.6	24.7	23.7	24.00	18.7	18.6	18.4	18.7	18.9	18.66

The figures indicate the diameter of inhibition zones assayed by pulp disc method in mm.

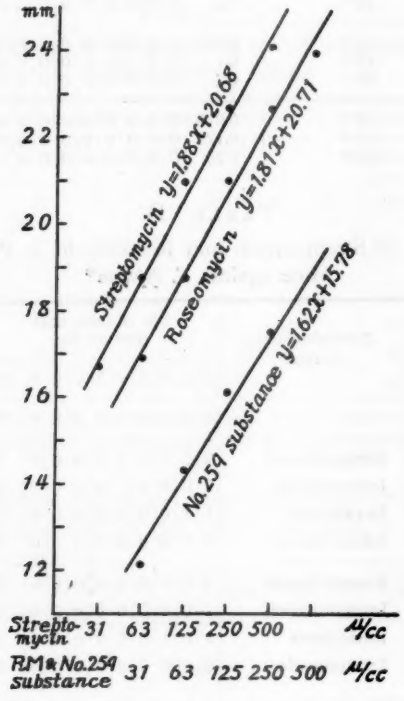

Fig. 1. Diffusibilities of 3 basic antibiotics

biotics, as shown in Table II.

As shown in Table III, in vivo activities were compared with 3 antibiotics. The antibiotics were administered immedately after infection.

As the result almost same activity was proved with these 3, so the high activity of No. 259 in vitro was not confirmed in vivo.

The second in vivo test was conducted with streptomycin and roseomycin both administered 1 hour after the infection. The administration was made through 3 routes, as shown in Table IV. 8 times of dosage was required with both antibiotics, when compared with the former experiment. From the above experiment, it was revealed, that, though the in vitro activity of roseomycin against gram positive organisms is low, it has a remarkable effectiveness against gram negative organisms in vitro and in vivo.

Finally, diffusion activity of 3 antibiotics against typhoid bacilli was compared with each other, as shown in Table V and Fig. 1. Least diffusibillity of No. 259 substance appeared to be closely correlated with the in vivo inactivity of it.

SUMMARY

The activities of 3 antibiotics, streptomycin, roseomycin and No. 259 substance were tested against *S. typhosa* both in vitro and in vivo. The effectiveness of streptomycin and roseomycin has been proved especially in vivo.

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Comparative Study of the Effect of Many Basic Antibiotics on *Hæmophilus pertussis* and on Experimental Murine *Pertussis*

(Studies on the Antibiotic Substances from *Actinomyces*, 12th Report)

By

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Many attempts were made to treat whooping cough both chemotherapeutically and immunologically, Streptomycin[1] demonstrated its ability to protect experimental infections of mice, and aerosporin[2] showed a remarkable effect in experimental infections and in clinical cases.

In this laboratory many streptothricin-like basic substances,[3] effective against a number of gram-negative organisms, were isolated, so the present studies were undertaken to examine the activities of these substances against *H. pertussis* in vitro and against experimental murine pertussis, being compared with those of streptomycin and "colistin"[4] which was supplied from Kobayashi Pharmaceutical Co., Ltd. "Colistin" is an aerosporin-like substance produced by a spore-forming bacteria and purified by the same method with that of aerosporin.

Experimental

Materials

As basic substances from streptomyces roseomycin (Nos. 36 and 20), 259 and "Seki" substance were used. The former two belong to so-called streptothricin type 1 (ST 1), characteristic in their low toxicity, in relatively low inhibitory action and in a good diffusibility, and the latter two belong to so-called streptothricin type 2 (ST 2) characteristic in their high inhibitory action, in relatively high toxicity and less diffusibility. All these substances were used as HCl-salt.

Results

1) Against 6 strains of *H. pertussis*, these substances were effective

TABLE I

Effects of 6 Antibiotics against 6 Strains of *H. pertussis* in vitro

Antibiotics	Tested strains	Conc. of HCl-salt (mg/cc)										Antibiotics	Tested strains	Conc. of HCl-salt (mg/cc)									
		1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120			1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120
Streptomycin HCl-$CaCl_2$ double salt	1	−	−	−	−	−	±	+	+	+	+	No. 20 substance HCl-salt	1	−	−	±	+	+	+	+	+	+	+
	2	−	−	−	−	−	±	+	+	+	+		2	−	−	+	+	+	+	+	+	+	+
	3	−	−	−	−	±	±	+	+	+	+		3	−	−	±	+	+	+	+	+	+	+
	4	−	−	−	−	−	−	+	+	+	+		4	−	−	+	+	+	+	+	+	+	+
	5	−	−	−	−	−	−	+	+	+	+		5	−	−	±	+	+	+	+	+	+	+
	6	−	−	−	−	−	−	+	+	+	+		6	−	−	±	+	+	+	+	+	+	+
Roscomycin HCl-salt	1	−	−	+	+	+	+	+	+	+	+	No. 259 substance HCl-salt	1	−	−	±	±	+	+	+	+	+	+
	2	−	±	+	+	+	+	+	+	+	+		2	−	−	+	+	+	+	+	+	+	+
	3	±	±	+	+	+	+	+	+	+	+		3	−	−	+	+	+	+	+	+	+	+
	4	−	±	+	+	+	+	+	+	+	+		4	−	−	+	+	+	+	+	+	+	+
	5	−	−	±	+	+	+	+	+	+	+		5	−	−	±	+	+	+	+	+	+	+
	6	−	±	±	+	+	+	+	+	+	+		6	−	−	±	+	+	+	+	+	+	+
"Seki" substance HCl-salt	1	−	−	−	±	±	+	+	+	+	+	Colistin HCl-salt	1	−	−	−	−	−	−	−	−	±	+
	2	−	−	−	±	±	±	+	+	+	+		2	−	−	−	−	−	−	−	±	+	+
	3	−	−	−	±	±	+	+	+	+	+		3	−	−	−	−	−	−	−	±	+	+
	4	−	−	−	−	+	+	+	+	+	+		4	−	−	−	−	−	−	−	±	+	+
	5	−	−	−	−	+	+	+	+	+	+		5	−	−	−	−	−	−	−	−	+	+
	6	−	−	−	−	+	+	+	+	+	+		6	−	−	−	−	−	−	−	−	+	+

Medium: 10% blood agar. The drugs were diluted serially 2 fold by this medium, starting from the concentration 1:10. Strain: No. 1 Kambayashi; No. 2 Sakairi; No. 3 Minada; No. 4 Tokunaga; No. 5 Shimada; No. 6 Koseki strain. All the strains were isolated from patients. Reading were made by the macroscopic examination, after 48 hours incubation at 37°C. +=visible growth. ±=slight growth. −=no visible growth.

in the following order: colistin, streptomycin, "Seki" substance, No. 259, No. 36 and No. 20 substances. The inhibitory potencies of all these substances were shown in Table I. Among 6 strains any resistant one was resistant to all these basic agents, whereas any susceptible one susceptible to all of the drugs. The susceptibility of *H. pertussis* to streptomycin and streptothricin-like substances was nearly the same with that of *E. coli*, while the former organism were more susceptible to colistin than *E. coli* (Table II).

TABLE II

Activity Ratio of 6 Antibiotics against *E. coli* and *H. pertussis* in vitro

Antibiotics	Minimum inhibitory dosage (mg/cc)		Activity ratio *E. coli*: *H. pertussis*
	against *E. coli* (Umezawa strain)	against *H. pertussis* (Kambayashi strain)	
Streptomycin	$\frac{1}{160}$ mg.	$\frac{1}{160}$ mg.	1 : 1
Roseomycin	$\frac{1}{16}$	$\frac{1}{20}$	1 : 1
"Seki" substance	$\frac{1}{80}$	$\frac{1}{160}$	1 : 2
No. 20 substance	$\frac{1}{16}$	$\frac{1}{20}$	1 : 1
No. 259 substance	$\frac{1}{80}$	$\frac{1}{40}$	2 : 1
Colistin	$\frac{1}{320}$	$\frac{1}{1280}$	1 : 4

TABLE III

Effect of 3 Antibiotics in Protecting Mice against Intranasal Infection of *H. pertussis*

No. of mice	Antibiotics	Daily dosis	Duration of Treatment	No. of mice that died on day										Death rate	Mean of significance (%)
				1.	2.	3.	4.	5.	6.	7.	8.	9.	10.		
5	Streptomycin	2.5 mg.	5 days	1	0	0	0	0	0	0	0	0	0	1/5	2.4
5	Colistin	0.6	5	0	1	0	0	0	0	0	0	0	0	1/5	2.4
5	"Seki" substance	2.5	5	0	0	1	3	0	0	0	0	1	0	5/5	
5	Control			1	1	3								5/5	

Infection: 4 mg. of culture of *H. pertussis* intranasally.

Treatment: First Injection of drugs was made intramuscularly 4 hours after infection. The daily dosis was divided into 4 and injected every 6 hours.

2) For the experimental infection in mice, both intranasal and intracerebral inoculation methods were used. The former method needed a relatively large amount (4 mg.) of organisms, in order to kill all control animals within 5 days, while the latter method required a relatively small number of organisms, i.e. 1/500 mg., to cause the death of animals within 9 days. Using both these methods it was clearly shown that streptomycin

TABLE IV

Effect of 3 Antibiotics in Treating Mice against H. *pertussis* Infected Intracerebrally

No. of mices	Antibiotics	Daily dosis	Duration of treatment	No. of mice that died on day														Death rate	Mean of significance(%)
				1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.		
5	Streptomycin	2.5 mg.	5	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1/5	2.4
5	Colistin	0.6	5	0	0	0	0	1	0	1	0	0	0	0	0	0	0	2/5	8.3
5	"Seki" substance	2.5	5	0	0	0	0	2	0	0	3							5/5	
5	Control			0	0	0	2	1	1	1									

Infection: 1/500 mg. (0.04 cc. of 1/20 mg. suspension per cc.) dose of H. pertussis culture were given intracerebrally. Treatment started 12 hours after infection. The daily dosis was divided into 4 and injected intramuscularly.

and "Colistin" difinitely protect against the experimental infection (Table III and IV).

SUMMARY

Streptomycin, colistin and several streptothricin-like substances were tested against *H. pertussis* both in vitro and in vivo.

Many kinds of streptothricins were ineffective, and the most favourable results were obtained with streptomycin and colistin.

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Clinical Evaluation of Rotatory Cross Section Radiography (Rotatory Crossgraphy) applied to Chest Diseases

(Studies on Rotatography. 11th Report)

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Since Rotatory Cross Section Radiography was invented by Vallebona,[1] Gebauer,[2] or Takahashi,[3] it has been hoped that this method can be applied to the full field of clinical medicine. However, there have not been yet published sufficient data as expected. Studying on this theme since December 1949, we have had a pretty wide experience and have come to a conclusion about the evaluation of this method. We will detail about the theme below.

Technique and Materials

584 patients, suffering from chest disease, were taken normal roentgenograms. Then they were rotatory-crossgraphed by means of the same apparatus and the technique in the former issue of this Journal.[4] And 867 roentgenograms, revealing some significant lesions in the chest, were obtained.

The ages of the patients ranged from three to seventy years.

Results

The diagnosis of the disease was established first with the normal roentgenogram, then that diagnosis was checked with the Rotatory Cross Section Radiogram.

If the latter found the additional findings and contributed a better diagnosis, this method was estimated as useful. We classified these incidences into four groups as follows.

A. Group applied to detecting lesions

126 out of 867 roentgenograms were estimated as useful in detecting lesions (Tab. I).

TABLE I

Name of lesions detected	Cases
1. Cavitation surrounded by dense consolidations	21
2. Cavitation hidden by shadow of the clavicula, ribs or scapulae	12
3. Intrapulmonary lesions surrounded by pleural lesions	17
4. Lesions situated in the vicinity of or in mediastinum superior	
anterior	14
posterior	11
5. Intrapulmonary lesions hidden by the heart displaced or dilatated	11
6. Paravertebral lesions hidden by the diaphragmatic arch	5
7. Lesions of the hilum	19
8. Lesions hidden by the mediastinum displaced by artificial pneumothorax, thoracoplasty or old pleurisy	16

There were three types of lesions in this group, namely, the one hidden by an extrapulmonary shadow, the intrapulmonary one surrounded by a dense shadow and the one situated at the inlet of the mediastinum. The first one might be found out easily in an oblique or a lateral view of normal roentgenography. The second one would be discovered of its true nature by means of tomography too.

Nevertheless the third one localized either in or in the vicinity of the upper mediastinum or paravertebral lesion hidden by the diaphragmatic arch might not be found out by the tomographic procedure due to the occurrence of nucleus shadows. This area was made clear only by the Rotatory Cross Section Radiography.

B. Group applied to differential diagnosis

214 out of 867 cross section roentgenograms were useful to establish the proper diagnosis. (Tab. II).

TABLE II

Name of lesions differentiated	Cases
1. Cavitation imaged in a ring figure and suspected as superimposition of shadows	58
2. Lesions superimposed at the area of the hilum and differentiated as:	
lymphadenitis	17
perihilar infiltration and intrapulmonary lesions	12
3. Opacity with sharp border, differentiated as:	
encapsulated pleurisy	8
athelectasis	5
pneumonia	7
lobus accessorius inf.	2
4. Cases misdiagnosed as the intrapulmonary lesions and later proved to be:	
pleural callosum	11
encysted pleurisy	10
interlobare pleurisy	10

	pulm. lesion combined with pleurisy	18
	pulm. infiltration	8
5.	Enlargement of the upper mediastinum found due to:	
	anterior mediastinal pleurisy	24
	paratracheal lymphadenitis	12
	pulmonary tuberculous infiltration	3
	thymus hypertrophy	4
6.	Miscellaneous,	
	Stomach hernia or cold abscess etc.	5

This result would not have been obtained by an ordinary roentgenography only, because the differential diagnosis in this group has been difficult to be established until the location of lesions was made clear. For this purpose either the Rotatory Cross Section Radiography or the normal roentgenography in oblique or lateral view was suitable. The latter, however, would fail to image lesions of the mediastinum clearly.

There were contained other cases in this group in which ring shadow imaged on the pulmonary field of a normal roentgenogram was suspected as a mere superimposition of line shadows. This was differentiated too as a image of cavitation when that was imaged as a ring shadow on both roentgenograms which were taken by normal roentgenography and by Rotatory Cross Section Radiography.

C. Group applied to the determination of the extent and position of lesions (Table III)

505 out of 867 roentgenograms were useful to determine the site, extent or position of lesions.

TABLE III

	Name of lesions made clear of their location etc.	Cases
1.	Cavitation	72
2.	Tuberculous infiltration	282
	The one accompanied by the atrophy of the thorax	21
3.	Hilusinfiltration	45
4.	Pleurisy,	
	costal	27
	encysted	4
	interlobar	3
	serothoracic	6
5.	Artheriosclerosis	26
6.	Aneurysma	2
7.	Aortic valvular disease	7
8.	Mitral valvular disease	10

Concerning the diagnosis these lesions had already been diagnosed properly with the normal roentgenogram. Either the state of the thoracic

atrophy or the deformity of the heart caused by the valvular diseases is manifested more cleary on occasion by this roentgenography. Moreover three dimensional knowledge about the lesions has never been learned by a normal roentgenographic procedure. From this point of view this method was estimated as useful.

D. Group applied to the pretherapeutic procedure

153 out of 867 roentgenograms served us not only in diagnostic use but also in therapeutic one (Tab. IV).

TABLE IV

Name of treatment of lesions, surgical or radiological	Cases
1. Collapstherapy in determining the state of a collapsed lung after:	
thoracoplasty,	44
extrapleural prombaging or pneumosewing	8
pneumothorax	37
2. Determination of the location of foreign body	6
3. Punction of the exudative pleurisy or the cavitation	16
4. x-ray treatment of lung tumors adjusted with isodose chart.	
pulmonary or broncheal tumor	25
metastatic tumor	9
mediastinal tumor	8

The collapsed lung adhered to the mediastinum in pneumothorax, or the one displaced to the retrovertebrale sinus in thoracoplasty was found out easily by this method.

The location of foreign bodies was most easily determined.

In planning the irradiation to the pulmonary tumor we applied the isodose chart to the cross section of the chest. Without the Cross Section Radiogram the reasonable irradiation would not be performed.

Discussion

As for profitable features of the Rotatory Cross Section Radiography, Takahashi,[4)5)] like Vallebona[6)] and Gebauer,[7)] has pointed out from his own experience that this method was superior to the customary roentgenography because the Cross Section Radiography enabled us to obtain the image of cross section of the body, and it was supperior also to tomography because that method made clear the state of the mediastinum. This is now proved here clearly. Moreover the merits of this method, when applied to chest diseases, were enumerated one by one. Concerning its ability in detecting lesions except the one in the vicinity of the mediastinum, the Rotatory Cross Section Radiography may be a little inferior to tomography due to its fuzzy images, but it can show more directly the location

and the shape of lesions in the cross section.

In differentiating the diagnosis of illness, this method serves us just as effectively as the oblique view of the normal roentgenography.

It was learned actually that the real diagnosis is to be established upon the acknowledgment of the location or the shape of lesions in the chest.

Moreover this methode has advantage that it can be applied to the area of pretherapeutic procedure.

The roentgenograms taken with our technique (small inclination of central x-ray of 15° to the film) reveals the state of the upper pulmonary field free from the obstructive shadow (or line shadow[8]) of ribs, which is often seen in the image taken with other techniques than ours.

Thus at least as regards the lesions located in the apex or in the vicinity of the mediastinum our technique can be said to be more effective than other techniques. A pretty big number of 584 of the patients upon which our experiments were based seems to strengthen our conclusion.

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On Pig-Liver Medium and a Modified Medium For Cultivation of *Bacterium Tularense*

By

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Hiwatashi[1] devised pig-liver medium for cultivation of *Bact. tularense* in 1943 and with this medium we[2] succeeded in isolating *Bact. tularense* directly from the pathological lymph nodes of Yatobyo patients (Ohara's disease) and from the infected animals. The efficiency of pig-liver medium and one of its modification, and the results of direct isolation of the germ by using them are reported.

Efficiency of the Solid Media Experimented by Inoculation with Dilution of the Cultivated Germ

Materials and methods. The strain of *Bat. tularense* used in the experiment had been isolated in May, 1950, from a lymph node of a Yatobyo patient, Ebina, in this laboratory. It had the virulence of killing white mice, weighing 15–18 g., within about a week with 10^{-8} mg. of it.

Method of preparation of pig-liver medium. 500 g. of pig-liver was cut into small pieces and mushed, to which 1000 cc. of neutral distilled water was added and heated at 100°C. for an hour or two in Koch's Sterilizer to make an extract. The extracted fluid was first filtered through gauze and then by filter-paper. 5 g. of natrium chloride and 10 g. of pepton were added to 1000 cc. of this filtered extract and this preparation was sterilized in an autoclave. Basic medium was thus secured.

To this medium, 3 per cent of agar, 0.1 per cent of cystine and 1 per cent glucose were added, sterilized at 100°C. for 30 minutes and, after being adjusted to pH 7.4–7.6, the fluid was filtered, divided in tubes and sterilized at 100°C. for 30 minutes three times. And then, when the heat came down to 45°C., 10 per cent of rabbit blood was added to each tubes, and the slant medium was made. We used 4 per cent sodium hydroxide to dissolve cystine easily. The end point of pH of the medium was from 6.8 to 7.0. We used the medium after incubating it at 37°C for 48 hours and after non-existence of saprophytes had been confirmed.

Preparation of modified pig-liver medium. Basic medium was as same

as above-mentioned. The required quantity of the basic medium was prepared in flask A, to which 2 per cent of dextrose, 0.3 per cent of cystine and 4 per cent agar were added, and it was autoclaved for 20 minutes at 115°C. This fluid was adjusted to pH 8.0, filtered and autoclaved for 20 minutes at 115°C. In flask B the same quantity of the basic medium was prepared, to which 1 per cent of hemoglobin (Takecho) was added. The hemoglobin was first made into an uniform paste, which, after about two hours, was filtered through gauze and then adjusted to pH 7.0 and autoclaved at 115°C for 20 minutes. The rest of the steps were as same as those taken in Steinhaus' liquid medium.[3][4]

Namely, the above-mentioned two preparations were cooled between 50°C and 60°C, thoroughly mixed, divided, 10 cc. each, into medium-sized test tubes, and coagulated so as to slant.

The pH of the water of condensation was between 6.8 and 7.0.

Experiment. Sufficient growth from a 48-hour-old culture of *Bact. tularense* (strain Ebina) was taken from the surface of a pig-liver medium to produce the basic suspension, in which 1 mg. of the organisms were suspended in 20 cc. of physiological salt solution. From this serial ten-fold dilutions in salt solution were made to the end point of 10^{-10}.

Duplicate tubes of both types of media then inoculated with 0.2 cc. quantities of the various dilutions of organisms from 10^{-5} mg. to 10^{-12} mg. Then the tubes were incubated at 34°C.

We observed daily for ten days at the same hour. The results of the growth are shown according to the rule given in Table I.

TABLE I

Signs for the Results of Cultivation

Sign	Condition of growth of germ
###	Colonies developed over two-thirds of surface of a slant
##	Colonies developed between one-third and two-thirds of surface of a slant
++	Colonies developed within one-third of surface of a slant, or number of colonies over 11
+	Number of colonies under 10
−	No growth

Results. As Table II shows, organisms developed on pig-liver medium even when it was inoculated with 10^{-9} mg. dilution of culture. On the other hand, they stopped developing on the modified medium when it was inoculated with 10^{-6} mg. dilution of culture.

TABLE II

Results of Cultures as Shown by Dilution of Germs

Dilution \ Type of medium / Day of culture	Pig-liver							Modified pig-liver						
	1	2	3	4	5	6	7	1	2	3	4	5	6	7
10^{-5} mg.	−	−	++	###	###	###	###	−	−	+	++	###	###	###
10^{-6} mg.	−	−	++	###	###	###	###	−	−	−	−	−	−	−
10^{-7} mg.	−	−	+	###	###	###	###	−	−	−	−	−	−	−
10^{-8} mg.	−	−	−	++	###	###	###	−	−	−	−	−	−	−
10^{-9} mg.	−	−	−	++	###	###	###	−	−	−	−	−	−	−
10^{-10} mg.	−	−	−	−	−	−	−	−	−	−	−	−	−	−
10^{-11} mg.	−	−	−	−	−	−	−	−	−	−	−	−	−	−
12^{-12} mg.	−	−	−	−	−	−	−	−	−	−	−	−	−	−

Results of Direct Isolation of *Bact. tularense*

Results on pig-liver medium

Materials and methods. Materials used in the experiment were 7 cases of lymph nodes which were removed from Yatobyo patients at the surgical department of our hospital, between December, 1951 and July, 1952. These were fresh materials.

The other 6 cases of lymph nodes, which were sent from other hospitals at several parts of Fukushima prefecture had shown 3 to 5 days' lapse before experiment.

Direct inoculation. In cases in which suppration in lymph nodes had not yet taken place, a piece of tissue was taken, put into the tube and rubbed on the surface of the medium, and in cases in which suppration had occured, pus taken from foci was rubbed with a loop on the medium.

Mouse inoculation. A piece of tissue taken from the removed pathological lymph node was ground in a mortar and made into 5-fold emulsion by adding salt solution, 0.2 cc. of which was inoculated into mice subcutaneously. Several days after inoculation, the dead animals were autopsied, and then their heart blood or spleen were inoculated on the solid medium.

Results. From all these 13 materials *Bact. tularense* could be isolated successfully.

As regards methods of cultivation, as is shown in Table III, we succeeded in 11 out of 13 cases by direct inoculation, while by animal inoculation we were successful in all the 13 cases.

As regards freshness of materials, the organisms could be isolated from all the materials obtained in our hospital, but of those sent from

TABLE III

Results of Experiment

Source of material \ Mode of experiment	Direct inoculation		Animal inoculation	
	number of cases	number of successes	number of cases	number of successes
Our hospital	7	7	7	7
Other hospitals	6	4	6	6
Total	13	11	13	13

other hospitals direct isolation was possible in 4 out of 6 cases. Namely, in cases of direct inoculation, isolation of the germs was easily influenced by freshness of materials. But in cases of animal inoculation, isolation was possible in all cases.

That is to say, when animals were used, the results were not so much influenced by freshness of materials.

In regard to the kind of materials being used, the results were as shown in Table IV.

TABLE IV

Results Classified by Pathological Sources

Mode of experiment	Direct inoculation				Animal inoculation			
Pathological sources / Source of material	Pus		Tissue of lymph nodes		Heart blood		Spleen	
	number of cases	number of successes	number of cases	number of successes	number of cases	number of successes	number of cases	number of successes
Our hospital	4	4	7	6	7	7	6	5
Other hospitals	4	1	5	3	6	6	6	5
Total	8	5	12	9	13	13	12	10

TABLE V

Days Required for Isolating the Germ

Mode of experiment	Direct inoculation		Animal inoculation	
Material	Pus	Tissue of lymph node	Heart blood	Spleen
Number of cases	5	13	13	10
Average days	5.8	5.9	2.4	2.4

N. B. In case of animal inoculation 5 to 10 days elapsed till animals died.

The number of days required to isolate *Bact. tularense* was as shown in Table V. In cases of animal inoculation, 5 to 10 days were required after inoculation till the inoculated animals died, and, 2 to 4 days were required to isolate the germ on solid media. It required one to two weeks in all cases for germs to be isolated.

Results on the modified medium

We had no opportunity till now to cultivate the germ directly from human materials on this modified pig-liver medium.

But in 11 out of 12 cases the germ was successfully isolated, from heart blood of infected mice inoculated with *Bact. tularense* by using this modified medium.

In only one case cultivation was unsuccessful, because of saprophyte growths.

Results of subculture on these media

By using these two solid media, we made subculture of over 30 strains, which had been stored in a refrigerator about one month. No difference was seen between the results exhibited by both media, and abundant growths of germs were obtained at 34°C from 24 to 48 hours cultivation.

Discussion and Summary

It is not very difficult to obtain direct isolation of *Bact. tularense* from human lymph-node tissue or pus, if suitable media are used.

We have successfully isolated the germ from human materials of 13 cases, all of which were obtained during the past eight months, by using pig-liver medium (Hiwatashi).

When it comes to the comparision of the two methods, i.e., direct inoculation and animal inoculation, only two cases were found negative in direct cultivation. Namely, when the materials were fresh, direct inoculation produced fairly good results.

Concerning the materials used, pus, which has heretofore been considered to be less suitable than lymph-node tissue for the purpose of isolating the germ, proved to be as suitable as the latter, because the results were positive in all cases with fresh pus.

However, when it was not fresh enough, the results of isolation were poor. By using spleen of inoculated animals, which obviously contains abundant germs and so should be the most suitable material for direct isolation of this germs, pure culture can often be disturbed by saprophytes.

The time required to isolate the germ was from 4 to 8 days by the direct method, and one to two weeks by the animal inoculation method, because several days had to pass till the animals were dead.

In conclusion, it is desirable to use concurrently the two methods, i.e., direct and animal inoculations, even when material is fresh.

Next, when the results on pig-liver medium (Hiwatashi) and the modified one are compared, pig-liver medium is far more suitable to culture *Bact. tularense*, than the modified one, observing the fact that the germ still grows even in dilution of 10^{-9} mg.

But, in case of subculture, both media can be useful, and when the germ is to be isolated from heart blood of infected mice, the results out of the two media will show little, if any, difference.

Therefore, the modified medium is sufficiently usable as subculture medium to preserve strains. Moreover, this semisynthetic solid medium is convenient to prepare and its materials can be obtained cheaper than the other's.

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Comparative Study of Roseomycin, Streptomycin, Chloramphenicol and Homosulfanilamide on the Effect of Experimental Cholera Infections

(Studies on the Antibiotic Substances from *Actinomyces*, 13th Report)

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Ishida of our laboratory has isolated a new basic streptothricin-like, but non-toxic substance " roseomycin[1] " from a strain of *Streptomyces roseochromogenus*. This substance showed a higher activity against *Vibrio comma* than streptomycin in vitro.

The author made a comparative study, dealing with the activities of roseomycin, streptomycin, chloramphenicol, sulfathiazole and homosulfanylamide against *V. comma* in vitro and of roseomycin, chloramphenicol and homosulfanilamide in mice inoculated with this organism.

Experimental

Materials and Methods

1) Roseomycin: HCl salt by chromatographic procedure. Purity of it was estimated as 98% of standard.

2) Chloramphenicol: given by Dr. Umezawa, m.p. 150°C.

3) Sulfathiazole: made by Kanebo and Co.. (m.p. 200–202°C)

4) Streptomycin: HCl-$CaCl_2$ double salt made by Merck and Co.. (770 u/mg)

5) Homosulfanilamide: made by Takeda and Co. (m.p. 256–260°C).

6) Culture media for *V. cholerae*.

Routine nutrient agar plate pH 7.5. The drugs mentioned above diluted serially by the medium and 10 strains of *Cholera vibrio* were streaked on the plate.

7) Method of animal infection and treatment: Mice weighing 16-23 g. were injected with 0.5 cc. suspension containing 10^{-2}–10^{-3} mgs. of 24 hour culture of Inaba strain in 5% mucin intraperitoneally and 1

TABLE I

Inhibitory Activity of Several Kinds of Antibiotics against *Vibrio comma*

Drugs	Strains tested	Concentration mcg/ml									
		50	25	12.5	6.3	3.1	1.6	0.8	0.4	0.2	0.1
Roseomycin (HCl-salt)	1	−	−	±	+	+	+	+	+	+	+
	2	−	−	±	+	+	+	+	+	+	+
	3	−	−	+	+	+	+	+	+	+	+
	4	−	−	+	+	+	+	+	+	+	+
	5	−	−	±	+	+	+	+	+	+	+
	6	−	−	±	+	+	+	+	+	+	+
	7	−	−	+	+	+	+	+	+	+	+
	8	−	−	+	+	+	+	+	+	+	+
	9	−	−	±	+	+	+	+	+	+	+
	10	−	−	±	+	+	+	+	+	+	+
Chloramphenicol	1	−	−	−	+	+	+	+	+	+	+
	2	−	−	−	−	+	+	+	+	+	+
	3	−	−	−	±	+	+	+	+	+	+
	4	−	−	−	+	+	+	+	+	+	+
	5	−	−	−	+	+	+	+	+	+	+
	6	−	−	−	+	+	+	+	+	+	+
	7	−	−	−	+	+	+	+	+	+	+
	8	−	−	−	+	+	+	+	+	+	+
	9	−	−	−	±	+	+	+	+	+	+
	10	−	−	−	−	+	+	+	+	+	+
		1000	500	250	125	62.5	31.3	15.6	7.8	3.9	2.0	0.1
Streptomycin (HCl-$CaCl_2$ double salt)	1	+	+	+	+	+	+	+	+	+	+	+
	2	+	+	+	+	+	+	+	+	+	+	+
	3	+	+	+	+	+	+	+	+	+	+	+
	4	+	+	+	+	+	+	+	+	+	+	+
	5	+	+	+	+	+	+	+	+	+	+	+
	6	+	+	+	+	+	+	+	+	+	+	+
	7	+	+	+	+	+	+	+	+	+	+	+
	8	+	+	+	+	+	+	+	+	+	+	+
	9	+	+	+	+	+	+	+	+	+	+	+
	10	+	+	+	+	+	+	+	+	+	+	+
Sulfathiazole	1	−	−	−	±	+	+	+	+	+	+	+
	2	−	−	−	±	+	+	+	+	+	+	+
	3	−	−	−	±	+	+	+	+	+	+	+
Homosulfanilamide	1	−	−	−	±	+	+	+	+	+	+	+
	2	−	−	−	±	+	+	+	+	+	+	+
	3	−	−	−	±	+	+	+	+	+	+	+

Strain tested: 1. Inaba, 2. Ogawa, 3. Hikoshima, 4. Miura, 5. Ri, 6. Gokampo, 7. Go. Pa, 8. 170, 9. 178, 10. 174.

hour after the infection all 4 drugs shown in Table II were injected subcutaneously into the back of each of animals in the amounts indicated.

Results

1. Against 10 strains of *Vibrio comma*, these substances were effective

TABLE II

The Therapeutic Experiment of *Vibrio comma* Infection in Mice with Roseomycin, Chloramphenicol, Homosulfanilamide and Streptomycin

Infection (mg., infected intraperitoneally)	Treatment after one hour of infection			No. of mice	Results						
	Drugs	Injection method	Single dose		No. of mice that died on day					Death rate	Mean of significance (%)
					1.	2.	3.	4.	5.		
10^{-3}	Roseomycin	Subcutan.	0.05mg.	5	3	0	0	0	0	3/5	9.1
			0.1	"	1	0	0	0	0	1/5	0.1
			0.3	"	2	0	0	0	0	2/5	1.7
10^{-2}		"	0.5	"	1	0	0	0	0	1/5	0.04
			1.0	"	1	0	0	0	0	1/5	0.04
10^{-3}	Chloramphenicol	"	0.05	"	2	0	0	0	0	2/5	1.7
			0.1	"	2	0	0	0	0	2/5	1.7
			0.3	"	1	0	0	0	0	1/5	0.1
10^{-2}		"	0.5	"	1	0	0	0	0	1/5	0.04
			1.0	"	2	0	0	0	0	2/5	0.4
10^{-3}	Homosulfanilamide	"	0.5	"	4	0	0	0	0	4/5	20.0
			1.0	"	2	0	0	0	0	2/5	1.7
			3.0	"	2	0	0	0	0	4/5	1.7
10^{-2}		"	5.0	"	2	0	0	0	0	2/5	0.4
			10.0	"	3	0	0	0	0	3/5	3.3
10^{-3}	Streptomycin	"	1.0	"	4	0	0	0	0	4/5	66.6
		Intraperiton.	1.0	"	4	0	0	0	0	4/5	66.6
10^{-3}	Control			20	19	0	0	0	0	19/20	
10^{-2}				20	20	0	0	0	0	20/20	

in vitro in the following order: chloramphenicol, roseomycin, sulfathiazole, homosulfanilamide, and streptomycin (Table I). Chloramphenicol and roseomycin were effective in a dose of 12.5 and 25 mcg. respectively.

2. In experimental infections, roseomycin and chlorapmhenicol showed a similar favourable effect i.e., both drugs were curative in a dose of 0.5–1 mg. against cholera infection caused by 10^{-2} mg. of *Cholera vibrio* in mice (Table II).

SUMMARY

The effectiveness of roseomycin in vitro and in experimental cholera infections as well as that of choloramphenicol has been proved. On the contrary streptomycin was proved to be ineffective.

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InVitro and Vivo Activities of Several Kinds of Basic Antibiotics from Streptomyces upon *Pasteurella Tularensis*

(Studies on the Antibiotic Substances from *Actinomyces*, 14th Report)

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The activities of several kinds of basic antibiotics[1] from streptomyces isolated in the author's laboratory were tested upon *Pasteurella tularensis* in vitro and in vivo, being compared with those of streptomycin, chlororamphenicol and colistin[2] (Aerosporin like substance).

Experimental

Materials and Methods

Streptomycin (hydrochloride, calcium chloride double salts), roseomycin-HCl salt (streptothricin type 1), 0-20-HCl salt (streptothricin type 1), Seki-substance-HCl salt (streptothricin type 2), No. 259-HCl salt (Streptothricin type 2), chloramphenicol (crystal m.p. 151°C) and pruified Colistin-HCl salt were used.

As test medium for *Pasteurella tularensis* was used a nutrient broth containing 1% glucose, 1% cystine and 8% horse serum. All the drugs mentioned above were added to this medium in concentration of 1:10,000 and were diluted 2-fold serially starting from this concentration.

For the inoculation of each tube a suspension of *Pasteurella tularensis*, cultivated on egg yolk-serum culture media at 37°C for 72 hours, in concentration of 5 mg./3 cc. was used in amount of 0.3 cc. The inhibition of the growth was determined after incubation at 37° C, for 72 hours.

For the infection of animals several amounts of wet bacteria were suspended in 0.5 cc. of 5% mucin solution and injected intrapertoneally into mice weighing 15–20 g. By preliminary experiments it was decided that 1 mg. of bacteria was just sufficient to kill a mouse under the inoculation condition mentioned above. This dosis of bacteria was determined as inoculation dosis.

Results

1. The minimum amounts of these substances required to inhibit *Pasteurella tularensis* in vitro were: Sterptomycin (3 mcg./cc.), roseomycin (25 mcg./cc.) and 0–20 substance (50 mcg./cc.). All other substances, such as chloramphenocol, Colistin, No. 259 and Seki substances showed no activities at all in concentration of 100 mcg./cc. (Table I)

TABLE I

In Vitro Activity of Several Antibiotics against *Pasteurella tularensis*

Antibiotics	Dilution (×10.000)									
	1	2	4	8	16	32	64	128	256	Control
Streptomycin	−	−	−	−	−	−	±	+	+	+
Roseomysin	−	−	−	±	+	+	+			++
0–20 (ST 1)	−	−	+	+	+	+				+
Seki Substance (ST 2)	+	+	+	+	+	+	+			+
No. 259 Substance (ST 2)	+	+	+	+	+	+	+			+
Chloramphenicol	+	+	+	+	+	+	+	+	+	+
Colistin (aerosporin-like substance)	+	+	+	+	+	+	+			+

(−) no growth, (±) minimum growth, (+) growth, (++) good growth.

TABLE II

Preliminary Test on the Activity of the Activity of Roseomycin, 0-20 Substance and Streptomycin in Vivo

Antibiotics	No. of mice that died on day					Death rate
	1.	2.	3.	4.	5.	
Control	4	1	0	0	0	5/5
Streptomycin	2	0	0	0	0	2/5
Roseomycin	0	0	0	1	0	1/5
0–20–substance (Streptothricin type 1)	2	0	0	0	0	2/5

2. A preliminary test was conducted by intraperitoneal injection of each of 3 antibiotics in 10-fold dosages of the minimum inhibitory concentration, i.e. 31 mcg. of streptomycin, 250 mcg. of roseomycin and 500 mcg. of 0–20 substance respectively 1 hour after intrapertoneal inoculation with 1 mg. of living bacteria (Table II). It was proved that the activity of roseomycin and streptomycin was excellent.

3. In order to compare the effect of roseomycin and streptomycin in a same dose 100, 250, 500 and 750 mcg. of both preparations were

injected intraperitoneally one hour after infection with 1 mg. of living bacilli. As shown in Table III, both streptomycin and roseomycin were proved to be remarkably and similarly effective.

TABLE III

Comparison of the Activity of Roseomycin with that of Streptomycin

Antibiotics	Single dose (mcg)	No. of mice that died on day										Death rate	Mean of significance (%)
		1.	2.	3.	4.	5.	6.	7.	8.	9.	10.		
Control	0	10	10	0	0	0	0	0	0	0	0	20/20	
Streptomycin	100	3	0	0	0	0	1	1	0	0	0	5/5	
	250	1	0	0	0	0	1	0	0	0	2	4/5	20
	500	0	0	0	0	0	0	1	0	1	1	3/5	3.3
	750	1	0	0	0	0	0	0	1	0	0	2/5	0.4
Roseomycin	100	3	0	0	0	0	0	2	0	0	0	5/5	
	250	0	0	0	2	1	1	0	0	0	0	4/5	20
	500	1	0	0	0	0	0	0	1	0	0	2/5	0.4
	750	1	0	0	0	0	0	0	0	2	0	3/5	3.3

The treatment was given only one time after injection.

TABLE IV

Comparison of the Activity of Roseomycin with that of Streptomycin

Antibiotics	Single dose (mcg)	Total times of injection	No. of mice that died on day										Death rate	Mean of significance (%)
			1.	2.	3.	4.	5.	6.	7.	8.	9.	10.		
Streptomycin	100	4	2	0	0	0	0	0	0	0	0	0	2/5	2.1
	250	4	0	0	0	0	0	0	0	0	0	0	0/5	0.03
	500	4	1	0	0	0	0	0	0	0	0	0	1/5	0.4
	750	4	0	0	0	0	0	0	0	0	0	0	0/5	0.03
Roseomycin	100	4	2	0	0	0	0	0	0	0	0	0	2/5	2.1
	250	4	1	0	0	0	0	0	0	0	0	0	1/5	0.4
	500	4	0	0	0	0	0	0	0	0	0	0	0/5	0.03
	750	4	0	0	0	0	0	0	0	0	0	0	0/5	0.03
Control		0	8	2	0	0	0	0	0	0	0	0	10/10	

Treatments were given 4 times after injection.

4. Further, in order to confirm a more beneficial effect the injections of drugs were repeated 4 times in a same dose mentioned above (Table IV). The injections were performed after 1, 7, 13 and 25 hours from inoculation. An excellent effect of roseomycin was proved.

SUMMARY

The activities of several kinds of basic antibiotics were tested against *Pasteurella tularensis* both in vitro and in vivo.

In experimental tularemia infection in mice, both streptomycin and roseomycin showed a similar excellent preventive effect in spite of different activities in vitro.

References

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2) Koyama, Y., Kurosawa, A., Tsuchiya, A. & Takakuta, K., J. Antibiotics (Japa.), 1950, **3**, 457.